THE ROLE OF S100A8 AND S100A9 PROTEINS IN THE PANCREATIC AND COLORECTAL CANCER MICROENVIRONMENT

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November 2012

Dedicated to my wonderful parents Mumtaz and Masood, my lovely wife

Farheen, and most beloved Zainab

For their endless love and support throughout my career

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PART ONE: INTRODUCTION

ABSTRACT

Introduction: Pancreatic cancer is a challenging solid organ malignancy, which despite advancements in surgery and oncology carries a dismal outcome. It is characterised by a dense desmoplastic stroma accounting for its bulky fibrotic nature. Recent years have seen the focus shift from the tumour cells as direct drivers of carcinogenesis to the wider tumour microenvironment. The distinct molecular 'cross-talk' between cancer cells and the surrounding desmoplastic stroma has emerged as a key mediator of carcinogenesis, affecting angiogenesis, invasion, metastasis and evasion of the host defences. It has been previously demonstrated through 2-DE analysis of laser capture microdissected malignant and stromal components of pancreatic tumours that high levels of S100A8 and S100A9 are expressed in tumour-associated stroma but not in malignant epithelial cells. It has also been demonstrated that SMAD4-negative pancreatic tumours contain fewer stromal S100A8-positive cells than their SMAD4-positive counterparts. The aims of this project were to unravel the association of S100A8 and S100A9 with tumour cells and elucidate their role in pancreatic cancer tumorigenesis.

Methodology: Protein expression (S100A8 and S100A9) was validated using a pancreatic cancer tissue microarray by immunohistochemistry and co-immunofluorescence methods, identifying the presence of these proteins in $CD14^+/CD68^-$ monocytes/macrophages. The effects of conditioned media from pancreatic cancer cells on the expression of S100A8 and S100A9 was also evaluated using western blotting. Recombinant GST-tagged S100A8 and S100A9 proteins were generated in *E. Coli* and purified using a glutathione-sepharose column. The effects of these proteins on the motility and proliferation of pancreatic and colorectal cancer cell lines, including SMAD4 negative cell lines in which SMAD4 has been re-expressed was accessed using modified Boyden Chamber Assays and MTT assays. The presence of TGF-β and $CD68^+$ macrophages was evaluated in the pancreatic cancer microenvironment and associations with S100A8/A9-expressing monocytes determined.

Results: The treatment of cultured monocytes (primary and cell lines) with conditioned medium from pancreatic cancer cell lines induced the expression of S100A8 and S100A9 proteins in them. Possible mediators of this effect included TGF-β and VEGF-A, the presence of which was identified in the conditioned media collected from pancreatic cancer cell lines and in tissue specimens (for TGF-β). Low levels of TGF-β expression in the tumours

significantly correlated with reduced number of S100A8-positive monocytes (p=0.04) and moreover high levels of tumour nuclear TGF-β expression correlated to a poor 2-year survival (p = 0.039). Incubation with GST-S100A8 and GST-S100A9 on their own or in combination significantly increased pancreatic and colorectal cancer cell motility by up to 10-fold compared to the GST protein control which varied based on the type of cell line used. Proliferation of the colorectal cancer cells was significantly increased in a dose-dependent manner with GST-S100A8 and GST-S100A9, with the highest increase in proliferation occurring between 36 and 48 hours of incubation. Treatment of SMAD4 positive and negative re-expressing colorectal cancer cells did not demonstrate any differential chemotaxis or growth.

Conclusion: These experiments have unravelled a relationship between cancer cells and S100A8 and S100A9 proteins in the pancreatic cancer microenvironment. There is a direct effect of pancreatic cancer cells on the expression of S100A8 and S100A9 in monocytes in vitro. These proteins significantly increase pancreatic and colorectal cancer cell motility and proliferation. Certainly further studies to evaluate the mechanisms of S100A8/A9 signalling in the tumour microenvironment will shed light on how these proteins influence the processes of tumour development and spread providing opportunities for targeted intervention.

STATEMENT OF ORIGINALITY

I declare that this is original work performed in the Department of Molecular and Clinical Cancer Medicine, University of Liverpool between May 2006 and August 2008. Except when stated, I have performed all the experimental work and data interpretation. This work would not have been possible without the technical assistance of the following individuals:

Dr Sarah Tonak: helped in the generation of recombinant S100A8 and S100A9 proteins

Dr Fiona Campbell: jointly scoring the tissue microarray for TGF-β staining

Mr Andrew Dodson: performed immunohistochemistry and immunofluorescence

Mr Chin Ang: jointly performing some of the motility and proliferation assays, where stated

Adnan Ahmed Sheikh **November 2012**

ACKNOWLEDGMENTS

I would like to take this opportunity to acknowledge the numerous people whose invaluable guidance and support have made this work possible. First and foremost, I would like to acknowledge my supervisor Dr Eithne Costello for her invaluable support, guidance and patience, teaching me many key skills and concepts which I will utilize thought out my career. I would like to thank Mr Andrew Dodson for helping me with immunohistochemical and immunofluorescence studies and Dr Fiona Campbell for her valuable time in analysing slides and scoring the tissue microarrays.

I would like to thanks all my co – workers, Liz, Khaled, Julie, Taoufik and Mark who put up with all the noise I made in the laboratory but were still willing to help me whatever the task no matter how big or small. A very special thanks to Sarah whose exceptional guidance throughout my MD made a lot the seemed impossible, possible and Bill Greenhalf for his great advice in particular statistical and for his humour. I want to thank Chin for all his help in the motility and proliferation work we did together and for his early morning runs to the lab for the time course experiments. I would like to thank Professor John Neoptolemos not only for all of his help, but also for giving me the opportunity to perform this work and for introducing me to academic surgery. Finally, no acknowledgement is complete without thanking my wife, Farheen and daughter Zainab for their love and support throughout my work.

Adnan Ahmed Sheikh **November 2012**

FUNDING

The studies described in this thesis were undertaken between May 2006 and August 2008 whilst employed as a Research Registrar within the Division of Surgery and Oncology, University of Liverpool.

Financial support was generously provided by the Royal Liverpool University Hospital for the first year of my studies.

For the second year of my studies, I gratefully acknowledge the generous support of the Grand Lodge of Freemasons, 250th Anniversary Fund for funding my Research Fellowship at the Royal College of Surgeons of England.

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

The Royal College of Surgeons of England

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LIST OF ABBREVIATIONS

2DE	Two dimensional electrophoresis
APS	Ammonium persulphate
CC	Chemokines
CDK	Cyclin dependant kinases
CSF	Colony stimulating factor
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylendiaminetetraacetic acid
EGF	Epidermal growth factor
FAMMM	Familial Atypical Multiple Melanoma
FAP	Familial adenomatous polyposis
FGF	Fibroblast derived growth factors
FPC	Familial pancreatic cancer
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
H&E	Haemotoxylin and eosin
HCl	Hydrochloric acid
H. pylori	Helicobacter pylori
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
ICAM	Intercellular adhesion molecules
IMPN	Intraductal papillary mucinous neoplasm
MMP	Matrix metalloproteinase

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAGE	Polyacylamide gel electrophoresis
PanIN	Pancreatic intrepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet derived growth factor
PRSS1	Trypsinogen gene
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
STK11	Serine / threonine kinase 11 mutation
TAMs	Tissue associated macrophages
TGF-β	Transforming growth factor beta
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TNF-α	Tumour necrosis factor alpha
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

REAGENTS USED

Unless otherwise stated, all reagents used were standard laboratory grade reagents.

Accetic acid

Acrylamide

Agarose

Ammonium persulphate

Ammonium sulphate

Beta mercaptoethanol

Bromophenol blue

Coomassie brilliant blue-G250 dye

EDTA

Eosin

Ethanol

Formaldehyde

Glycerol

Glycine

Hydrochloric acid

Magnesium sulphate

Methanol

Protease inhibitor cocktail tablets (Roche pharmaceuticals)

SDS

Silver nitrate

Sodium acetate

Sodium carbonate

Sodium hydroxide

TEMED

TRIS

Trypsin

Water

Xylene

PART ONE: INTRODUCTION

CHAPTER ONE

BIOLOGY OF PANCREATIC CANCER

1.1 Pancreatic Cancer

Incidence

Pancreatic ductal adenocarcinoma (PDAC) (referred in the text also as pancreatic cancer) is amongst the most challenging of solid organ malignancies, being the 5th leading cause of cancer related deaths in the developed world owing to its propensity for late presentation and chemo resistant tumour biology (Chua and Cunningham, 2006; Siegel et al., 2012; Vincent et al., 2011). In the UK, pancreatic cancer is ranked 11th accounting for over 7000 newly diagnosed cases per annum (Cancer Research, 2009; Neoptolemos, 2011), with a worldwide incidence of nearly a quarter of a million newly diagnosed cases (Ghaneh et al., 2007; Vincent et al., 2011). The American Cancer Society estimates, over 40000 newly diagnosed pancreatic cancer cases in the USA alone (Siegel et al., 2012) (Society, 2010) with Europe having over 60,000 new cases per year (Ghaneh et al., 2007). The International Agency for Cancer Research (IARC) estimates that there will be over 230,000 new cases of pancreatic cancer and about 227,000 deaths worldwide due to this devastating disease (IARC, 2008). Moreover, European cancer mortality prediction figures for 2012, indicate that pancreatic cancer mortality rates are likely to increase by 2-3% this year with all other tumours, except lung cancer, demonstrating a decrease in cancer related mortality (Malvezzi et al., 2012).

Despite improvements in the field of patient management and a better comprehension of the cytogenetics of this disease, the overall 5-year survival is still dismal at less than 5% (Buchholz and Gress, 2009; Vincent et al., 2011). Without treatment, metastatic

pancreatic cancer carries a grave prognosis, with median survival between 3-5 months (Wray et al., 2005). This is not significantly better for locally advanced disease, which is 6-10 months (Buchholz and Gress, 2009) (Ghaneh et al., 2007). Surgery remains the main stay of treatment, however owing to the late presentation and aggressive nature of this cancer only 10-15% of patients are deemed operable at the time of presentation (Neoptolemos, 2011; Tuveson and Neoptolemos, 2012; Wray et al., 2005). Data from clinical trials indicate that chemotherapy enhances survival in both patients undergoing surgery and in those with advanced disease (Cunningham et al., 2009; Neoptolemos et al., 2004; Neoptolemos et al., 2009). Those patients undergoing resections and adjuvant chemotherapy have a median survival of approximately 23 months with a 5-year survival ranging between 10-20% (Ghaneh et al., 2007; Neoptolemos, 2011; Thomas et al., 2010).

Advancement in the field of molecular research allowing for a better understanding of pancreatic cancer tumour biology have made way for novel therapeutic targets which work in conjunction with current therapies enhancing their efficiency (Matsuo et al., 2012; Moore et al., 2007; Vincent et al., 2011). In addition, research into predictive biomarkers of response to standard therapies have also led to improved outcomes in patient cohorts (Costantino et al., 2009; Richards et al., 2010) with the ultimate aim being to provide tailored therapeutic regimes for patient with pancreatic cancer based on their tumour cytogenetics. Improvement in clinical services, particularly centralisation of care and a multidisciplinary team approach to perioperative management have also

contributed towards improved survival in the last two decades with in hospital mortality decreasing by up to 6 fold in some reports (Alexakis et al., 2004; Lemmens et al., 2011). There is substantial geographical variation in the incidence of this disease with the highest incidence seen in northern countries such as Iceland, Finland and the USA along with countries closer to the equator such as Egypt, Tunisia, Zimbabwe and India (Agbunag C and Bar-Sagi D, 2004; Raimondi et al., 2009). This incidence in these areas has remained constant over the last 3 decades in contrast to that in Western Europe, which has seen a steady rise (de Braud et al., 2004; Ghadirian et al., 2003). There exists a tenfold difference in both incidence and mortality rates when comparisons are made between countries with the highest and lowest risks for pancreatic cancer (Raimondi et al., 2009). The incidence is generally lower in women, although it is almost equal to that of men in groups that are exposed to additional risk factors such as alcohol consumption and smoking (Silverman DT et al., 2003; Wahi et al., 2009). One possible hypothesis for a lower incidence in females is the protective effect oestrogen is thought to have on pancreatic cancer (Wahi et al., 2009). The lowest prevalence of pancreatic cancer is observed in Africa and South Asia, although Japan is an exception with a high incidence reported (Ghadirian et al., 2003; Kinoshita et al., 2007). Populations in several Polynesian groups including Maoris and native Hawaiians are known to have a high incidence (Ghadirian et al., 2003) with Nigerians demonstrating a very low incidence of the disease.

Environmental risk factors primarily cigarette smoking (Lee and Hamling, 2009a; Lee and Hamling, 2009b; Porta et al., 2009) have been attributed towards the high incidence of this disease making it equal in both sexes. A significant difference is seen in age-

adjusted incidence of pancreatic cancer in global cancer registries (Raimondi et al., 2009). This variance is probably caused by different exposures to lifestyle and environmental factors or both, since careful genetic studies in twins predict that about two-thirds of pancreatic cancer is related to environmental factors (Lichtenstein et al., 2000). Similarly, epidemiological studies comparing the incidence of disease amongst Japanese migrants to the USA found it to be far higher than in their native Japanese family members (Ghadirian et al., 2003).

The disease has preponderance for age with an increasing incidence after 75 years, affecting 2 per 100,000 / year between 40-44 years compared to 67 per 100000/year in patients over 75 years of age. The 5–10% of patients who develop pancreatic cancer before the age of 50 years, are likely to include patients with underlying predisposing genetic disorders or those who have undergone previous treatments for cancer, such as radiotherapy (Raimondi et al., 2007) . Certain race and ethnic groups, e.g. Jews in Europe and African Americans are at an appreciably higher risk for developing pancreatic cancer compared to white or Asian populations. The reasons for this are unclear, although various nongenetic risk factors, such as smoking, diabetes, body mass index, and vitamin D insufficiency have been suggested (Raimondi et al., 2009; Silverman DT et al., 2003) (Ghadirian et al., 2003).

Aetiology and Risk Factors

Pancreatic cancer like other malignancies is multi-factorial in aetiology and heterogeneous in its development arising from a multitude of genetic and epigenetic defects. A better comprehension of the aetiology and molecular events over the last two decades has been instrumental in further understanding, management and screening of this devastating disease. Environmental and genetic risk factors for pancreatic cancer are further detailed in the sections below.

Smoking

Smoking is one of the primary risk factors for developing pancreatic cancer conferring a 70-100% increased risk (Iodice et al., 2008). This risk does gradually diminish, after cessation of smoking but does not return to baseline before at least 10 years (Iodice et al., 2008; Lowenfels and Maisonneuve, 2006). Tobacco products such as cigars and chewing tobacco exert a moderate increase in risk of pancreatic cancer with borderline significance (Alguacil and Silverman, 2004; Lowenfels and Maisonneuve, 2006). Though the exact mechanisms of the risk incurred by smoking is not yet clear, it is thought to be as a result of nitrosamines contained in nicotine. These are considered to be genotoxic in their effects, causing the induction of mutations in genes such as *KRAS* (Porta et al., 2009; Schuller HM, 2002). Crous-Bou *et al.* analysed mutations in codon 12 of the KRAS oncogene have challenged this hypothesis and lifetime consumption of tobacco in patients with pancreatic cancer, to conclude that smoking does not play a major part in the acquisition of *KRAS* mutations in the pancreatic epithelium (Crous-Bou et al., 2007). More recently, a meta-analysis of clinical and epidemiological studies concluded that a

lifetime history of tobacco consumption was not significantly associated with the frequency of *KRAS* mutations in pancreatic cancer (Porta et al., 2009).

Diet

Dietary factors seem to influence the development of pancreatic cancer and account for a several fold increase in the incidence of this disease in various countries (Lowenfels and Maisonneuve, 2006). There is some data to suggest that a diet high in vegetables and fruit is protective with a few of case control studies reporting a positive association with increased meat and cholesterol intake and pancreatic cancer development (Vincent et al., 2011) (Jiao et al., 2009) (Howe GR and Burch JD, 1996). However, no strong link between the development of pancreatic cancer and diet has yet been established. A large cohort study involving over half a million subjects in 10 European countries failed to find any protective benefit from fruit and vegetable consumption (Vrieling et al., 2009). Folates confer a lower risk, but data on this is inconsistent (Skinner HG et al., 2004) as is that on Vitamin D which in some studies is shown to have a protective effect on the development of pancreatic cancer (Giovannucci, 2009). Moreover, prospective and experimental studies have shown that obesity itself confers an increase risk of pancreatic cancer (Khasawneh et al., 2009) (Calle et al., 2003). The mechanism may be by direct tumour promotion or indirectly via obesity related dampening of inflammatory and protective responses (Khasawneh et al., 2009).

Alcohol and Coffee consumption

The role of alcohol in the aetiology of pancreatic cancer has been supported by retrospective, ecologic and cohort investigations (Ghadirian et al., 2003), however studies to the contrary have shown that moderate alcohol consumption does not increase the risk of developing the disease (Lowenfels and Maisonneuve, 2006; Rohrmann et al., 2009). Only high lifetime ethanol intake from spirits and liquor tended to be associated with a higher risk of developing PDAC (Rohrmann et al., 2009). The proposed mechanism of how alcohol increases the risk of PDAC is thought to be by induction of mutation in the *KRAS* gene which has been reported to be three times higher in both smokers and alcohol drinkers compared to non consumers with pancreatic cancer (Malats et al., 1997). Crous-Bou *et al.* analysed the lifetime history of alcohol consumption and *KRAS* mutations to conclude that alcohol consumption is only weakly associated with an increased risk of having a *KRAS* mutated PDAC. Furthermore, to confirm or refute the hypothesis that alcohol might influence the acquisition or persistence of *KRAS* mutations in the pancreatic ductal cells large and unselected studies are warranted (Crous-Bou et al., 2009). High alcohol intake itself leads to chronic pancreatitis which is a risk for developing pancreatic cancer (Ghaneh et al., 2007). The concept that coffee is a risk factor for pancreatic cancer has been challenged with recent data failing to demonstrate a significant association (Maitra and Hruban, 2008).

Diabetes Mellitus

Even though diabetes exists in approximately 10% of the population, the role of diabetes in pancreatic cancer has been investigated with conflicting results (Chari et al., 2008; Pandey et al., 2011; Raimondi et al., 2009). Everhart *et al.* undertook one of the initial meta-analysis examining 20 epidemiological studies and revealed a causal link between diabetes and the risk of developing pancreatic cancer (Everhart and Wright, 1995). A further analysis (Huxley et al., 2005) showed a more modest relationship between long standing diabetes of greater than 5 years and pancreatic cancer (odds ratio 1.5) with the risk being higher for patients who had diabetes mellitus for less than 5 years (odds ratio 2.0). Other case control and prospective studies have also demonstrated an increased risk of pancreatic cancer in diabetics (Luo et al., 2007). One of the major concerns in all of these studies is the fact that diabetes mellitus was diagnosed shortly before or concomitantly with the cancer and therefore whether diabetes mellitus causes pancreatic cancer or arises secondary to cancer is a controversial issue (Lowenfels and Maisonneuve, 2006) (Raimondi et al., 2009). Both experimental and epidemiological evidence to support the argument on both sides exit. Islet cell dysfunction has been implicated as one of the underlying factors behind diabetes seen with pancreatic cancer (Saruc M and Pour PM, 2003). This dysfunction is a result of a primary alteration by a carcinogen, or secondary damage caused by the cancer cells themselves. A further theory proposes the production of diabetogenic substances by the cancer cells themselves, which early studies have revealed to be low molecular weight peptides detected in the serum of patients with pancreatic cancer (Basso D et al., 2002). Islet amyloid polypeptide (amylin) has been investigated by several groups and is thought to have a similar function (Saruc

M and Pour PM, 2003). The impact of therapy for diabetes on pancreatic cancer risk has been examined with Metformin, an oral antidiabetic agent, being associated with a decreased risk of pancreatic cancer (Currie et al., 2009). Studies have also identified the relationship of obesity which not only affects peripheral insulin resistance resulting in cancer related diabetes, but is also seen to significantly increase the risk of pancreatic cancer (Calle EE and Kaaks R, 2004; Luo et al., 2007). The jury is out as to whether diabetes is an actual risk factor for pancreatic cancer or is diabetes in pancreatic cancer a result of the disease itself.

Other medical associations

Previous gastrointestinal surgery in particular cholecystectomy is a risk factor with reports from clinical studies suggesting a relative risk of developing pancreatic cancer in the range of 1.2-2 (Lin Y et al., 2002). To the contrary a larger prospectively designed study by Schernhammer *et al.* involving over 100,000 patients indicated the actual risk of pancreatic cancer due to cholecystectomy to be very low (Schernhammer ES et al., 2002). A recent meta-analysis of 18 studies has concluded that individuals with a history of a cholecystectomy are at an increased risk of developing pancreatic cancer (Lin et al., 2012). Previous partial gastrectomy for benign peptic ulcer disease has also been reported to carry an increased risk of pancreatic cancer, the mechanisms of which are unclear, but an increased production of nitrosamines is one of the proposed mechanisms (Tascilar M et al., 2002).

Helicobacter pylori (H. pylori) infection as a possible risk factor for pancreatic cancer has also met with conflicting results with some studies quoting an odds ratio ranging from 1.87-2.1 (Lowenfels and Maisonneuve, 2006). A large population based study from Sweden concluded no association between H. pylori infection and the risk for pancreatic cancer in the total cohort, however in a subset of subjects who were non-smokers and low alcohol consumers a positive H. pylori serology was associated with an increased risk for pancreatic cancer (Lindkvist et al., 2008). A recent cumulative meta-analysis has concluded that there is no increased risk of pancreatic cancer with Helicobacter pylori (H. pylori) infection (Trikudanathan et al., 2012).

Other viral infective agents that are known to cause acute pancreatitis e.g. coxsackie, mumps, HIV are not known to cause pancreatic cancer (Lowenfels and Maisonneuve, 2006), however studies have detected a link between Hepatitis B / Hepatitis C virus infections and pancreatic cancer (El-Serag et al., 2009).

Industrial and Environmental Risk Factors

Studies have linked pancreatic cancer to several occupational groups; workers in mines, metal works, sawmills, chemical plants, rubber factories and the petrochemical industry are at an increased risk (Andreotti and Silverman, 2012). Exposure to chlorinated hydrocarbon compounds, pesticides, polycyclic aromatic hydrocarbons (PAHs), metals, nitrosamines, radiation, naphthylamine, and benzidine have been reported to be linked to pancreatic cancer with the strongest evidence being associated with chlorinated hydrocarbons and PAHs (de Braud et al., 2004) (Andreotti and Silverman, 2012).

1.2 Pancreatitis and its role in Pancreatic Cancer development

Chronic pancreatitis

There is a well-established link between inflammation and cancer such as gastritis in the cases of stomach cancer or hepatitis causing hepatocellular carcinoma. Chronic pancreatitis is recognised to confer a 15-25 fold risk for pancreatic cancer in some studies (Olson, 2011) (Malka et al., 2002) (Howes and Neoptolemos, 2002). Patients with chronic pancreatitis usually have disease for a duration greater than 20 years and as a result of this tend to have calcification, increasing the risk of complications (pseudo cysts, diabetes) (Howes et al., 2004). Exocrine organ dysfunction and pancreatitis are thought to promote carcinogenesis in part by promoting the local release of growth factors, cytokines and reactive oxygen species resulting in cell proliferation, disruption of cell differentiation and enhancing oncogene mutations (Carriere et al., 2011; DiMagno and DiMagno, 2009; Zavoral et al., 2011).

Analysis of specimens resected for chronic pancreatitis have revealed that over a third of patients have *KRAS* mutations in the pancreatic tissue, moreover the presence of pancreatic cancer precursor lesions PanIN (pancreatic intraepithelial neoplasia) were detectable in 63% of specimens. This further supports the association of pancreatitis as a risk factor for pancreatic cancer (Andea et al., 2003; Guerra et al., 2007). Mouse model experimentation has also shown that induction of pancreatitis in a 2 month old mouse causes rapid PanIN progression and PDAC development by the age of 4 months (Carriere et al., 2011).

The developmentally regulated family of Hedgehog genes also has a role in chronic pancreatitis and pancreatic cancer (Hidalgo and Maitra, 2009; Jones et al., 2008). The Hedgehog pathway is one of the core signalling pathways that undergoes alteration in pancreatic cancer (Hidalgo and Maitra, 2009). Likewise this pathway is also seen as unregulated in chronic pancreatitis (Kayed et al., 2004), reflecting its contribution towards pathogenesis. Mouse model experimentation has also shown upregulation of the Hedgehog pathway in rats with chronic pancreatitis (Wei-Guo et al., 2010).

Local factors produced in chronic pancreatitis in the way of proinflammatory cytokines (interleukins) and growth factors (epidermal and platelet derived) can cause activation of the NF-κB pathway (nuclear factor kappa-light-chain-enhancer of activated B cells) (Zavoral et al., 2011). The pathway is known to regulate cancer relevant processes including immune modulation, angiogenesis, and apoptosis (Zavoral et al., 2011). Normal pancreatic tissue does not demonstrate any NF-κB activity, however inflamed acinar cells and local stromal cells (such as macrophages) through autocrine and paracrine channels result in activation of this pathway modulating early pancreatic cancer pathogenesis (Chandler NM et al., 2004). Another downstream mediator of inflammation is the cyclooxygenase-2 (COX-2) which is a key enzyme in the production of prostaglandins and is seen to be up-regulated in pancreatitis and overexpressed in pancreatic cancer (Albazaz et al., 2005) (Zavoral et al., 2011). The association of a chronic inflammatory response in the pancreatitis microenvironment leads to tissue damage and fibrosis, which can also promote tumorigenesis by release of cytokines and growth factors, as well as release of reactive oxygen species (ROS) (Farrow and Evers, 2002). An increase in

intracellular reactive oxygen species can cause DNA damage, contribute to telomere shortening and mutation of proto-oncogenes, resulting in pancreatitis having a role pancreatic cancer tumorigenesis (Farrow et al., 2008) (Masamune et al., 2009b).

Hereditary pancreatitis

Hereditary pancreatitis confers a 70-100 fold-increased risk of developing pancreatic cancer (Haddad et al., 2011
; Howes N et al., 2004). Hereditary pancreatitis is an autosomal dominant disorder with an estimated penetrance of 80% (Ghadirian et al., 2003) and equal gender incidence, presenting in children and younger adults (Ghaneh et al., 2007) (Greer et al., 2009). EUROPAC, which is the European data registry for hereditary pancreatitis and familial pancreatic cancer, defines hereditary pancreatitis as two or more first degree relatives or three or more second degree relatives in two or more generations with recurrent acute pancreatitis and or chronic pancreatitis in the absences of gallstones, tropical pancreatitis or excess alcohol (Howes et al., 2004). It is characterised by recurrent attacks of acute pancreatitis that begin in childhood resulting in chronic pancreatitis by the age of 25 years.

A vital genetic mutation in hereditary pancreatitis was first described by Whitcomb *et al.* in 1996, who discovered a specific mutation on the cationic trypsinogen gene (PRSS1) in affected individuals from five kindred (Whitcomb DC et al., 1996). Up to 25 further mutations of the PRSS1 gene were subsequently described (Howes N et al., 2004) with

the R122H and N291 making 70% of those mutations (Greer et al., 2009). Mutated PRSS1 causes premature cationic trypsinogen gene activation resulting in excessive intracellular activation of trypsinogen to trypsin; the gain of function leading to chronic pancreatitis. The risk of developing pancreatic cancer is linked to the duration of chronic pancreatitis with cancer occurring 30 years after the onset of chronic pancreatitis, the incidence showing a very significant increase in smokers (Greer et al., 2009). Mutation in the pancreatic secretory trypsin inhibitor gene, SPINK1, has been associated with "tropical pancreatitis" which is seen in Asia and Africa conferring a risk of 100 fold with onset of cancer being about 14 years earlier than in sporadic cases (Whitcomb, 2004). Finally, there is a link between chronic pancreatitis and pancreatic cancer in patients with cystic fibrosis. Mutation in the CFTR gene causes impairment of enzyme secretion of pancreatic digestive enzymes resulting in ductal obstruction with pancreatitis leading to chronic fibrosis and ultimately cancer (Haddad et al., 2011

; Noone PG et al., 2001).

Inherited Genetics of Pancreatic Cancer

The inherited predisposition attributable to genetic factors accounts for 5-10% of observed cases of pancreatic cancer (Haddad et al., 2011

; Klein AP et al., 2004; Vincent et al., 2011). Germline mutation have been linked to familial pancreatic ductal adenocarcinoma including those targeting tumour suppressor genes: *INK4A, BRCA2* and *LKB1, MLH1* (Jaffee et al., 2002), however penetrance of these is low and they are seen to impact tumour progression rather than initiation (Hezel

et al., 2006). In certain families this has an associated autosomal dominant pattern of inheritance with the risk of developing pancreatic cancer amongst first degree relatives of an affected individual estimated to be 18 fold in kindreds' with two, and as high as 57 fold in kindreds' with three or more affected family members (Jorgensen et al., 2008). An inherited predisposition to pancreatic cancer has been suggested with some authors grouping them in syndromic and non-syndromic groups (Jorgensen et al., 2008). Commonly they care divided into three groups:

(1) As an adjunct to a familial cancer syndrome

(2) As an inherited predisposition to pancreatic cancer linked to another condition for example, hereditary pancreatitis

(3) As part of familial pancreatic cancer group where there is a predisposition to pancreatic cancer without a causative agent.

Familial pancreatic cancer

Familial pancreatic cancer (FPC) is rare in occurrence, being characterised by at least two first degree relatives or two or more second degree relatives (one of whom had an early onset below the age of 50 years) with histologically confirmed pancreatic cancer that does not fulfil the criteria of another hereditary cancer syndrome (Lynch et al., 1989) (Maitra and Hruban, 2008). Data from a prospective study suggested that the relative risk of developing pancreatic cancer among relatives of a pancreatic cancer patient was calculated to be 4.6 fold in kindred with one, 6.4-fold in kindred with two, and as high as

32-fold in kindred with three or more first degree family members (Jorgensen et al., 2008; Klein AP et al., 2004). The gender distribution for FPC is 43% men and 57% women, with a mean age of pancreatic cancer being 65 years (Lynch HT et al., 1990). The principle causative gene mutation has not been identified however up to 20% of families with familial pancreatic cancer have a *BRCA2* germline mutation(Hahn et al., 2003). Palladdin, a gene encoding for cytoskeleton proteins has recently been suggested as a candidate gene being identified in the susceptibility locus of 4q32-34 (Pogue-Geile et al., 2006) however these have not been seen in other large familial pancreatic cancer kindred (Earl et al., 2006).

Pancreatic cancer in a familial cancer syndrome

As described in the above, pancreatic cancer can occur as part of a defined genetic syndrome (Vitone et al., 2006) (Maitra and Hruban, 2008) (Greer et al., 2009). **Peutz-Jeghers syndrome**, which is a rare autosomal dominant condition, and poses the highest overall risk for pancreatic cancer with a 120-fold lifetime risk and cumulative lifetime risk of 36% (Giardiello FM et al., 2000). It consists of oral mucosal naevi associated with intestinal hamartomas due to serine / threonine kinase 11 (*STK11*) mutations (Grutzmann et al., 2004). In addition, it is associated with the development of cancer at multiple sites e.g. colonic cancer. **Hereditary non-polyposis colorectal cancer (HNPCC)** has an autosomal dominant pattern caused by mutations in *hMLH1* and *hMLH2* genes accounting for 90% of mutations (Peltomaki and Vasen, 2004). Aarnio *et al.* studied over 290 carriers in 40 families identifying 6 cases of pancreatic cancer making the overall

risk low (Aarnio et al., 1995; Jorgensen et al., 2008). **Familial ovarian and breast cancer** is caused by germline mutation in *BRCA1* or *BRCA2* genes, exhibiting a lifetime risk for developing pancreatic cancer in the order of 5% (Vitone et al., 2006). The *BRCA2* protein product interacts with proteins involved in cell cycle regulation, transcriptional regulation and DNA repair. Hereditary breast cancer is mainly caused by germline mutations in the *BRCA1* or *BRCA2* genes (Daniel DC, 2002). In a large multicenter study, Thompson *et al.* demonstrated that the relative risk for pancreatic cancer in *BRCA1* carriers was increased by 2.26 fold which was statistically significant (Thompson D and Easton DF, 2002). This relative risk in *BRCA1* carriers is lower than that in *BRCA2* carriers which has been estimated to range from 3.5 to 8 fold (Ozcelik H et al., 1997). **Familial adenomatous polyposis** is an autosomal dominant inherited diseases caused by germline mutation of *APC* (Adenomatous polyposis coli) resulting in thousands of colonic polyps appearing at an early age resulting in colonic cancer, however the reported incidence of pancreatic cancer in these patients is too small establish a definitive link (Offerhaus et al., 1992).

Pancreatic cancer is the second most common cancer that occurs in the **familial atypical multiple mole melanoma syndrome** and is particularly significant in patients and families with *p16* mutations (Vasen et al., 2000). In all the families with FAMM described so far the causative germline mutation has been found to be *INK4A*. **Melanoma-pancreatic cancer syndrome:** the joint occurrence of melanoma and pancreatic cancer in combination with a *CDKN2A* Germline mutation appears to be a distinct tumour predisposition syndrome, which has been termed melanoma-pancreatic

cancer syndrome (Bartsch DK et al., 2002). **Ataxia Telangiectasia** is an autosomal recessive syndrome that is associated with inactivation of the ataxia telangiectasia mutated *ATM* gene. The protein product has an important role in the cellular response to genetic stress inducing cell cycle arrest or apoptosis, by a *p53* independent pathway (Yang J et al., 2003). Carriers of the mutated gene have a relative risk of pancreatic cancer of approximately three-fold (Geoffroy-Perez B et al., 2001). The syndromes and associated genetic mutations, which are known to occur in them are summarised in Table 1.1.

Syndrome	Inheritance	Mechanism	Gene involved	Risk
Peutz-Jeghers Syndrome	Autosomal dominant	Serine / threonine kinase 11 mutations	*STK11/LKB1*	4.5 fold (Latchford et al., 2006)
Familial Breast and Ovarian Cancer	Germline mutation	Disruption of cell cycle and transcriptional regulation	*BRCA1* and *BRCA2*	2.26 fold(Thompson D and Easton DF, 2002)
Familial Atypical Multiple Mole Melanoma	Autosomal dominant	P16 inactivation	*CDKN2*	13 fold (Hezel et al., 2006)
Hereditary Non-polyposis Colon Cancer	Autosomal dominant	by mutations in DNA repair genes	*MLH1, MSH2, MSH6, PMS1, PMS2*	5 fold (Brentnall, 2000)
Ataxia-Telangiectasia	Autosomal recessive	Induction of cell cycle arrest	*ATM*	3 fold (Geoffroy-Perez B et al., 2001)
Fanconi Anaemia	Autosomal recessive	Repair of DNA damage	*FANCA* genes	5 fold (van der Heijden et al., 2004)
Li-Fraumeni Syndrome	Autosomal dominant	Germline mutations in p53	*p53*	2 fold (Ruijs et al., 2010)
Familial Adenomatous Polyposis	Autosomal dominant		*APC*	Low risk- 4.5 fold (Giardiello FM et al., 1993; Offerhaus et al., 1992)

Table 1.1: Table detailing familial cancer syndromes and associated genetic mutations

1.3 Molecular genetics of pancreatic cancer

The understanding of pancreatic cancer genetics and linkage of key molecular events to histopathologic stages is a rapidly evolving field, which has seen numerous developments recently (Vincent et al., 2011) (Iacobuzio-Donahue, 2011). Pancreatic cancer is a heterogeneous disease of inherited and somatic mutations however, the molecular mechanisms linking these genetic changes to the aggressive nature of this disease remains to be fully understood. Recent publications using advanced sequencing methods for mutational analysis of tumours and corresponding metastasis have given an in-depth insight into the genetic evolution of pancreatic cancer (Jones et al., 2008) (Campbell et al., 2010; Yachida et al., 2010). Moreover, studies mapping timeframes of tumours initiation, progression and kinetics of metastasis have revealed that these processes are considerably longer than previously thought to be (Haeno et al., 2012; Yachida et al., 2010).

Pancreatic cancer exhibits all the hallmarks of malignant cancer cells, including autocrine growth signalling, insensitivity to growth inhibition, evasion of lysis, angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2011; Iacobuzio-Donahue, 2011). These occur through complex biological pathways related to mutations, activation of proto-oncogenes and inactivation of tumour suppressor genes, abnormalities in growth factors and their receptors, gene amplifications, chromosomal losses and telomere shortening (Buchholz and Gress, 2009) (Mihaljevic et al., 2010
) (Haeno et al., 2012). This subsequently has a knock-on effect on downstream signal transduction pathways involved in the control of growth and differentiation.

Pancreatic cancer cells have certain signature molecular aberrations, which distinguish them from normal pancreatic ductal cells and are vital in maintaining the transformed phenotype of tumour cells. Jones *et al.* (Jones et al., 2008) in 2008 published a landmark paper delineating discovered aberrations to core signalling pathways in pancreatic cancer. The authors analysed 24 pancreatic tumours to a large-scale genomic analysis of 20,661 protein-coding genes, uncovering 1562 somatic mutations, 198 homozygous deletions and 144 amplifications. Thirty-one gene sets were identified as altered in a majority of the 24 pancreatic tumours analysed; these could be linked to 12 cores signalling pathways some of which have been studied in detail, whereas others have not previously been implicated in pancreatic cancer.

Molecular evolution of pancreatic cancer in terms of a temporal sequence of genetic alterations has revealed that a majority of genetic alterations occur early in the neoplastic process and are present in the primary tumour and metastases arising from it (Campbell et al., 2010). Using somatic mutations as molecular clocks, Yachida *et al.* were able to determine that an initial precursor neoplastic clone will take about 10 years to evolve into a malignant clone and several years for metastatic subclones to emerge from within the primary cancer (Yachida et al., 2010). This timeline is certainly longer than previously perceived and has improved prospects for therapeutic treatment and screening. The same group, using mathematical and computational analysis of primary pancreatic tumours and associated metastasis has more recently suggested that targeting tumour cells using chemotherapy whilst they were rapidly growing was crucial and surgery prior to

chemotherapy may reduce survival. This has challenged current practices triggering the need for further research, in particular novel clinical trials (Haeno et al., 2012).

Oncogenes

Oncogenes are dominant genes, which induce or maintain cellular transformation, which when mutated or expressed at high levels, help convert a normal cell into a tumour cell. Proto-oncogenes are present normally in cells and are involved in regulating cellular growth or differentiation (Vinay Kumar, 2007). Abnormal cells normally undergo a programmed form of death called apoptosis, however activated oncogenes can cause those cells to survive and proliferate instead. Mutations in proto-oncogenes result in unrestricted cell growth and this may be a result of (1) Point mutations (2) Chromosomal translocation (3) Gene amplification (4) Environmental factors (5) Viral infections (Vinay Kumar, 2007).

KRAS mutations

One of the fundamental oncogenetic mutations in pancreatic cancer is *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), which is reported to be mutated in 75% to 90 % of cases (Buchholz and Gress, 2009; Hruban et al., 1993) (Iacobuzio-Donahue, 2011). KRAS is 21 kDa protein (Ghaneh et al., 2007) and member of the RAS family of GTP-binding (Guanosine-5'-triphosphate) proteins that mediate a wide variety of cellular functions including proliferation, differentiation, cell survival and cytoskeletal remodelling amongst a few (Campbell et al., 1998; Hingorani and Tuveson, 2003;

Iacobuzio-Donahue, 2011). The active form of the protein is bound to GTP and inactivation occurs through guanosine triphosphate activating proteins (GTPase activating proteins), which promote hydrolysis to the diphosphate form (GDP). These activating mutations impair the intrinsic GTPase activity on the KRAS gene product, resulting in a protein that is constitutively active in intracellular signal transmission (Hingorani and Tuveson, 2003) leading to uncontrolled cell growth (Hruban et al., 2008). Activating *KRAS* point mutations occur at codon 12 resulting in the substitution of glycine with aspartate, valine, or arginine (Hruban et al., 1993). *KRAS* mutations are also demonstrable in the pancreatic cancer progression model, being present in up to 87% of cancer associated PanIN-2/3 lesions (Matthaios et al., 2011).The high frequency of *KRAS* mutation detected in PanINs support its function as an initiating event in the development of invasive disease (Hingorani et al., 2003; Hruban and Adsay, 2009; Matthaios et al., 2011). The other members of the *RAS* family (*HRAS* and *NRAS*) are not affected in pancreatic cancer (Rodenhuis, 1992). *KRAS* activation seem to be essential for pancreatic cancer maintenance as has been demonstrated by non-viability of dominant negative mutants cancer models and the use of interference siRNA knockdown studies (Fleming et al., 2005). Mice model experimentation has shown that the KRAS protein is required not only for initiation of pancreatic cancer but also for its maintenance suggesting a potential therapeutic target (Collins et al., 2012 1957). Posttranslational modification of RAS protein by C-terminus farnesylation through fernesyl transferase is a major therapeutic target using inhibitors of farnesyl transferase, however phase III trials have been unsuccessful (Rocha-Lima, 2008).

Activated *KRAS* is involved in three main effector pathways which are (1) RAF activated kinase (MAPK: Mitogen-activated protein kinase), (2) phosphoinositide-3-kinase (PI3K) and (3) RalGDS (guanine nucleotide dissociation stimulator) pathways.

The RAF family of serine / threonine kinase binds to activated KRAS, resulting in MAPK/ERK kinase activation through a series of phosphorylation culminating in proliferation and enhancement of a variety of intracellular systems (Hezel et al., 2006). Hirano *et al.* has demonstrated that inhibition of MAPK/ERK using pharmacological agents results in decreased proliferation and cell cycle arrest indicating the potential role of this pathway as a therapeutic target (Hirano et al., 2002; Neel et al., 2011). The inhibitor of RAF-1 kinase, Sorafenib (Kane et al., 2006) has been used successfully in practice to treat renal cell carcinoma. Sorafenib has limited activity in advanced pancreatic cancer (Matthaios et al., 2011). Isolated *B-RAF* mutations are present in about a third of pancreatic carcinomas, with a wild-type KRAS resulting in activation of RAF-MAPK signalling in the absence of *KRAS* mutation (Calhoun et al., 2003), indicating a *KRAS* independent pathway.

The *PI3K* signalling pathway, which can be activated by *KRAS*, regulates cell survival, size, and proliferation via several downstream effectors including AKT (serine/threonine protein kinase) , p70-S6K, and the small GTPase (Cantley, 2002; Rodriguez-Viciana et al., 1996). The PI3K-AKT pathway is activated in most pancreatic cancers via KRAS however there are genomic events which allow this to be activated independently namely via activation of the *AKT2* gene on chromosome 19q (Mihaljevic et al., 2010

). This gene has been demonstrated to be amplified and activated in up to 60% of pancreatic cancers (Altomare et al., 2003). The protein mTOR (mammalian target of rapamycin) a downstream regulator of PI3K and AKT pathway has emerged to have a role in *KRAS* mediated cell proliferation, which has been inhibited using CCI-779, causing growth inhibition, but is still under investigation as a form of therapy for pancreatic cancer (Javle et al., 2010).

Mutations of *KRAS* also affect downstream regulation of the RalGDS pathways through RalA, which has recently emerged as an important molecule in the regulation of *KRAS* mediated functions (Lim et al., 2006). Experimental work analysing the functioning of RalA and RalB discovered the former critical to tumour initiation, with the latter being important in tumour metastasis when tested in cancer cell lines (Neel et al., 2011). The potential of these proteins as future therapeutic targets in halting pancreatic cancer progression is yet to be identified (Neel et al., 2011).

The nuclear factor kappa B transcription factor (NF-κB) is another important downstream mediator of mutated *KRAS* signalling in pancreatic ductal adenocarcinoma being activated via the PI3 / AKT pathway. This pathway is activated in response to cell stresses by stimulation of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and growth factors having a role therefore in regulating immune response, apoptosis, and angiogenesis (Hayden and Ghosh, 2004). TNF-α is produced by a variety of cell including, macrophages as well as pancreatic acinar cells having a paracrine and autocrine effect on cancer cells. A majority of pancreatic cancer and cancer cell lines

demonstrate NFκB activity in the presence of *KRAS* activation as dominant negative *KRAS* alleles show no activity of NFκB (Liptay et al., 2003). The Figure 1.1 below demonstrates a simplified schematic representation of *KRAS* signalling in pancreatic cancer.

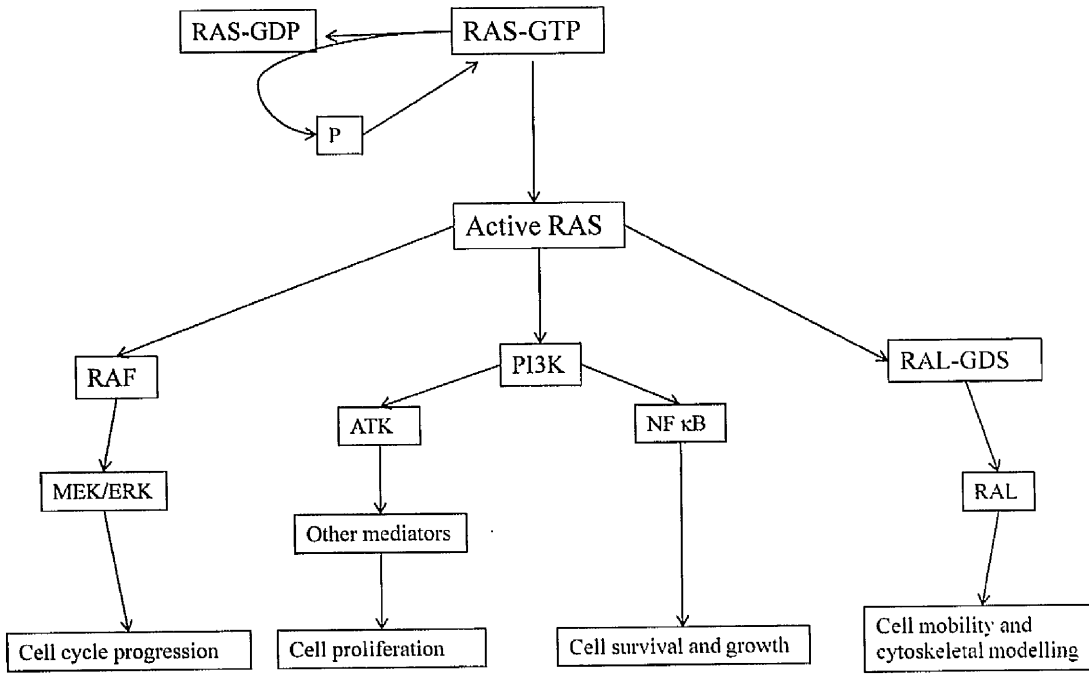


Figure 1.1: The RAS signalling pathway in pancreatic cancer. (Abbreviations of text in above section)

Tumour Suppressor Genes

Pancreatic cancer is associated with a high rate of inactivation of three tumour suppressor genes: *INK4A, p53* and *MADH/DPC4 (SMAD4)* (Tuveson and Neoptolemos, 2012). Tumour suppressor genes normally function in order to stop signals for cell division, with their activity going unchecked in carcinogenesis resulting in unregulated cell proliferation and growth. Unlike oncogenes, tumour suppressor genes generally follow the 'two-hit hypothesis', as the presence of one functional copy in most cases is sufficient to maintain the function of the gene (Knudson A, 1978). These tumour suppressor genes can be inactivated by a number of mechanism including homozygous deletion, intragenic mutations, or promoter hypermethylation (Hruban et al., 2008). Oncogene mutations, in contrast, generally involve a mutation to single allele, resulting in a single point mutation. There are notable exceptions to the 'two-hit' rule for tumour suppressors, such as certain mutations in the *p53* gene. Mutations of *p53* gene can function as a 'dominant negative', meaning that a mutated p53 protein can prevent the function of normal protein from the un-mutated allele occurs (Baker et al., 1990).

INK4A

The gene *P16/CDKN2A* (Cyclin-dependent kinase inhibitor-2A) also known as *INK4A* suffers a loss of function, which is brought about by mutation, deletion, or promoter hypermethylation and occurs in about 80%–95% of sporadic pancreatic ductal adenocarcinomas (Rozenblum et al., 1997; Vincent et al., 2011). In 40% of cancers homozygous deletion of both alleles of the gene (Hruban et al., 2008).

As a result, of either environmental carcinogens or instability of DNA, cell cycle fail safe mechanisms are activated to ensure the accuracy of each step of DNA synthesis is maintained. Depending on the phase in which damage is detected, non-transformed cells arrest in the G_1 (growth-1 phase), S (synthesis phase) or G_2 phase, (growth 2 phase) of the cell cycle allowing repair or apoptosis if repair is not possible to occur (Vinay Kumar, 2007). Loss of one or more of these cell cycle fail safe mechanisms results in uncontrolled proliferation of the cancer cells (Liu et al., 2010). Cyclin dependant kinase (CDKs) which form complexes with respective cyclin subunits (e.g. Cyclin-D and Cyclin-E) are at the heart of the cell cycle enabling phosphorylation of specific substrates and hence proliferation (Liu et al., 2010).

The *CDKN2A* locus (*9q21*) encodes two overlapping tumour suppressors *INK4A* and *ARF*, and their respective protein products $p16^{INK4A}$ and $p19^{ARF}$ via distinct first exons and alternative reading frames in shared downstream exons (Sherr, 2004). The close genetic proximity of both genes is the reason why genetic alteration affects both the tumour suppressor genes together on most occasions (Mihaljevic et al., 2010
). The function of the product of the *CDKN2A (INK4A)* gene, $p16^{INK4A}$ is to bind cyclin-dependant kinases CDK4/CDK6 thereby preventing the formation of active cyclinD-CDK4/CDK6 complex. The cyclinD-CDK4/CDK6 complex inhibits phosphorylation of Rb (retinoblastoma protein) which is a tumour suppressor protein (Sherr, 2004) (Liu et al., 2010). This in turn will block entry into the S phase of the cell cycle, which is the DNA synthesis phase (Maitra and Hruban, 2008). In pancreatic cancer, the Rb/INK4A tumour suppressor pathway is disrupted due *to INK4A* mutation resulting in uncontrolled

cell growth. The other tumours suppressor gene at the *CDKN2A* locus (*9q21*), ARF encodes for a product $p19^{ARF}$ that also inhibits cell proliferation being involved in the p53/MDM2 pathway. The protein $p19^{ARF}$ inhibits MDM2-dependent proteolysis of p53 resulting in activation of p53 hence Rb dependent cell cycle arrest (Sherr, 2006) (Hezel et al., 2006). Furthermore, *ARF* seems to have a *p53* independent tumour suppressor function by inhibiting NF-κB function and its anti-apoptotic activity (Rocha et al., 2003). Both mutation of *INK4A* and *ARF* gene occur simultaneously in around 40% of pancreatic cancers however, mutation targeting INK4A alone are commonly (Bardeesy and DePinho, 2002) observed as a major event in the development of pancreatic cancer. Bardeesy *et al.* has proven using mouse model experiments that *INK4A* mutation in co-operation with *KRAS* is paramount for the progression of pancreatic cancer producing tumours with notably less differentiation and a higher malignant and invasive potential (Bardeesy et al., 2006). Furthermore, *INK4A* abolition can accelerate tumour progression in the setting of concurrent mutation of *p53* (Bardeesy et al., 2006).

p53 Mutation and cell cycle regulation

The *p53* tumour suppressor gene is present at chromosome 17p being inactivated in over 50%-75% of pancreatic ductal adenocarcinomas, most commonly due to missence alterations of the DNA-binding domain (Iacobuzio-Donahue, 2011; Redston MS et al., 1994). This tumour suppressor gene plays an essential role in regulating cell growth and inactivation is seen to occur in the later-stages in PanINs (pancreatic intraepithelial neoplasia) and adenocarcinoma when the tumour is seen to have attained significant

dysplasia (Hruban and Adsay, 2009; Maitra et al., 2003) (Iacobuzio-Donahue, 2011). It is proposed that in more advanced PanINs genetic damage is more pronounced because of telomerase erosion and radical oxygen species hence allowing for *p53* elimination. This allow for cells harbouring genetic aberrations to grow in an uncontrolled manner (Maitra et al., 2006). Mutations in *p53* are crucial affecting the very core of the cell cycle. The p53 protein regulates the G1 to S phase of the cell cycle checkpoint and maintains the G2 to M arrest point (Maitra and Hruban, 2008). Loss of p53 function therefore serves to enable the growth and survival of cells harbouring procarcinogenic chromosomal aberrations. The p53 protein is also involved in the apoptotic pathway, the potential loss of these two important functions by inactivation or mutation of p53 may be a critical event in pancreatic cancer (Berrozpe G et al., 1994). As described previously in the *INK4A* section, the relationship between *ARF* and *p53* is important as *ARF* inhibits MDM2 mediated targeting of the p53 protein from degradation (Bardeesy and DePinho, 2002; Rozenblum et al., 1997). An ARF deficiency would result in marked reduction of p53 protein levels (Sherr, 2006) however in pancreatic adenocarcinoma *p53* mutations and *ARF* deletion coexists suggesting the non-overlapping function of these proteins (Maitra et al., 2003). *ARF* also has *p53* independent functioning such as inhibition of RNA processing resulting in apoptosis (Paliwal et al., 2006). A detailed explanation of regulation of the cell cycle is beyond the scope of this thesis, a brief account is described in the following paragraph and demonstrated in figure 1.2.

The cell cycle consists of four stages mitotic (M) phase, growth 1 phase (G1), synthesis phase (S) and growth 2 phase (G2) with cell division occurring the in M phase and DNA

synthesis occurring in the S phase(Vinay Kumar, 2007). The G1 and G2 phase provides additional checkpoints in which damaged DNA replication and cellular division errors are repaired (Bharadwaj and Yu, 2004). In the normal cell cycle the primary regulators are cyclin dependent kinase (CDKs) which attain an active kinase activity following binding with cyclin subunits which direct them (CDKs) towards phosphorylation of stage appropriate proteins (Liu et al., 2010; Maitra and Hruban, 2008). The CDK-cyclin complexes can be inactivated by removing the cyclin subunit, dephosphorylation, or by inhibition from a specific group of proteins called the cyclin dependant kinase inhibitors (CKI) which can potentially act as tumour suppressors (Liu et al., 2010). Many subsets of the CDK family have been identified in different phases of the cell cycle forming unique combinations of cyclins and CDKs at different stages of the cell cycle (Malumbres and Barbacid, 2005). Three different CDKs participate in the G phase (CDK2, CDK4, CDK6) whereas CDK1 is active in mitosis (M phase). The CDK4 and CDK6 are driven by the three D-type cyclins D1, D2 and D3 and cyclin-E, which specifically activates CDK2 (Malumbres and Barbacid, 2005). The primary target of the D-cyclin and to a lesser extent the E-cyclin is the retinoblastoma (pRb) gene product, a crucial regulator of cellular proliferation (Classon M and Harlow E, 2002). Phosphorylation of the pRb inactivates it, which results in it dissociation of Rb from transcription factors (E2F), leading to the generation of protein and DNA polymerases that are essential for DNA transcription at the G1/S phase transition (Hochegger et al., 2008). Mutation of the *Rb* gene is one of the first genetic alterations described in human cancers that leads to increased activation of E2F, unregulated transcriptional activation and uncontrolled cell growth (Johnson DG, 2000). It has also been seen in pancreatic cancer that mutation of

pRb expression and over-expression of E2F correlate with tumour grade (Hochegger et al., 2008).

The cyclin-CDK complex is inhibited by two classes of cyclin dependant kinase inhibitors in humans, the Cip/Kip family and the INK4A family (Liu et al., ; Sherr and Roberts, 1999). The Cip/Kip family is composed of three members: $p21^{WAF/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, which have a broad spectrum of activity, binding to cyclin-CDK complexes and inhibiting their activity. Besides regulating the cell cycle, Cip/Kip proteins play important roles in apoptosis, transcriptional regulation, cell fate determination, cell migration and cytoskeletal dynamics (Besson et al., 2008). The INK4A family is composed of p15, p16, p18 and p19, which more specifically disrupt CDK4 and CDK6 binding to it D-type cyclins in the G1 phase, therefore preventing phosphorylation of Rb protein as described previously.

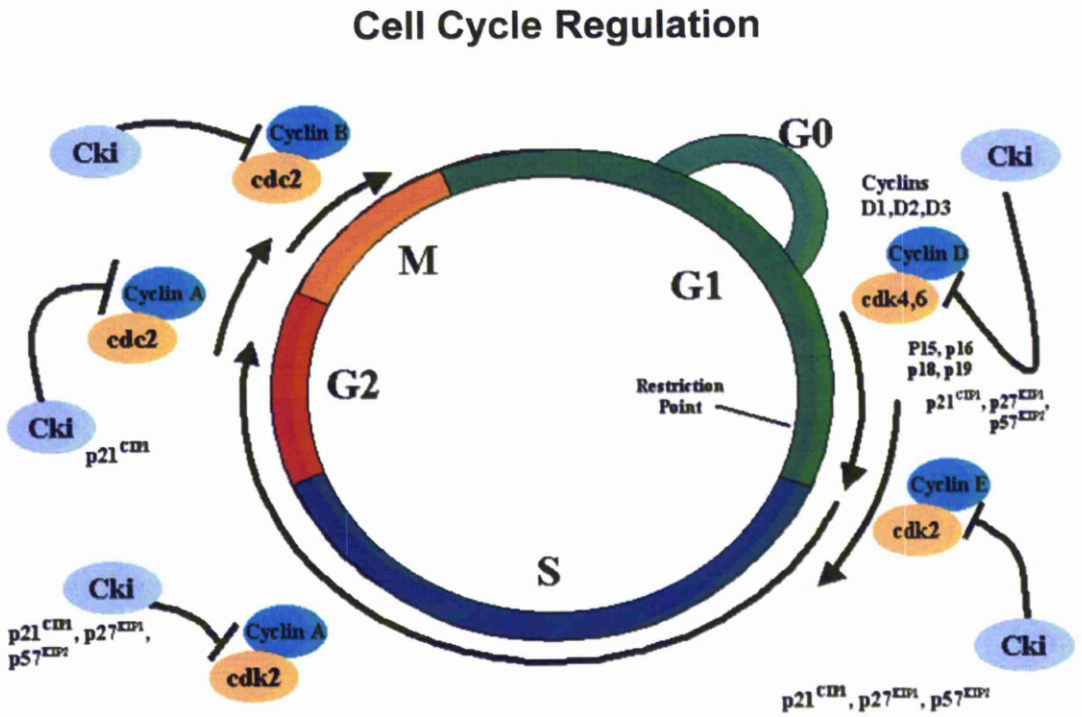


Figure 1.2: Schematic representation of Cell cycle with cyclin regulatory checkpoints

Developmental and signalling pathways

The understanding of the association of hedgehog and notch signalling pathways in pancreatic carcinoma has been established over the last decade shedding light on the embryologic development of the pancreas and pancreatic cancer (Maitra and Hruban, 2008). The section below briefly highlights the pathways involved, both biochemical and molecular which link pancreatic development to cancer.

Hedgehog

The hedgehog signalling pathway is important in pancreatic cancer growth and development which in the adult pancreatic tissue is turned off but reactivated in pancreatic cancer tumorigenesis (Hidalgo and Maitra, 2009). The mammalian family of hedgehog signalling pathway consists of three members: Sonic, Indian and Desert. Hedgehog works together with its transmembrane receptors partners 'patched' and smoothened allowing for signal transmission. Hedgehog pathway has a critical role in pancreatic cancer tumorigenesis as it is often mutated (Jones et al., 2008) and inhibition of this pathway with cyclopamine enhances survival in genetic mouse models (Feldmann et al., 2008) which occurs through direct interactions with Smoothened. The Sonic hedgehog signalling seems to be involved in pancreatic cancer stem cell viability (Jimeno et al., 2009) the existence of which is based on the hypothesis that cancers grow and propagate from a small proportion of cells which have properties similar to normal stems cell such as differentiation and renewal. Smoothened inhibitor IPI-926 has proven beneficial to survival when applied in conjunction with gemcitabine in a pancreatic cancer mouse model (Olive et al., 2009). This drug was able to achieve efficacy by

reducing fibrosis facilitating the distribution of gemcitabine. The hedgehog signalling pathway therefore seems linked not only to cancer and stem cell progression, but also to the microenvironment. There is evidence to suggest the hedgehog pathway is involved in pancreatic cancer initiation as the hedgehog ligand is over expressed in precursor lesion like PanINs and IMPN (Intraductal papillary mucinous neoplasm), along with chronic pancreatitis (Hidalgo and Maitra, 2009). Moreover it has been proposed in the literature that pancreatic cancer cell secrete hedgehog ligands activating the hedgehog pathway in the stroma promoting the dense desmoplastic response seen in pancreatic cancer (Hidalgo and Maitra, 2009).

Notch

The notch pathway has an important function in directing the processes by which precursor cells develop in the pancreas and in pancreatic cancer, initiation and invasion occurs (Ranganathan et al., 2011; Wang et al., 2011

). The Notch signalling pathway is activated by the binding of Notch ligands (delta-like and jagged) to one of the four Notch receptors (Notches 1-4)(Ranganathan et al., 2011). The interactions result in the proteolytic degradation of the Notch receptors, subsequent translocation and activation of target genes. Notch pathway is activated in PanIN lesions and invasive cancer where it is seen to promote vascularisation (Rehman and Wang, 2006). This pathway is also involved in mediating communication between adjacent cells expressing similar receptors and ligands being of importance in process of apoptosis differentiation and proliferation.

There is also evidence to suggest that Notch interacts with *KRAS* to initiate and maintain pancreatic ductal adenocarcinoma with the notch target gene *Hes1* seen to be elevated in precursor lesions in mouse models (Sundaram, 2005). Plentz *et al.* has shown that pharmacological blockage of the downstream Notch signalling pathway in pancreatic cancer cell lines results in decreased growth, and prevents pancreatic cancer formation in the mouse model (Plentz et al., 2009).

Telomere length abnormalities

The telomere is hexameric TTAGGG repeats at the end of chromosome arms, which allows for stability during cell division. Loss of telomeres results in chromosome breakage during anaphase (Meeker and De Marzo, 2004). This results in areas on the chromosomes that can develop regional amplification and deletions (O'Hagan et al., 2002). Telomere abnormalities are one of the commonest aberrations occurring in pancreatic cancer with over >90% of low grade PanIN lesions demonstrating shortening (Haeno et al., 2012; van Heek NT et al., 2002). As a result of telomere erosion in PanINs an environment is created which allows for acquisition of chromosomal rearrangement, however telomerase reactivation in invasive cancer allows for stability to invasive cancer cells (Sato et al., 2002). Chronologically it is known that telomere shortening precedes the loss of *p53* function and it is likely that telomere activates *p53* mediated suppressor pathways (Hruban et al., 2000; Vincent et al., 2011).

1.4 Pathogenesis of Pancreatic Cancer and Genetic Evolution

Morphology

Pancreatic ductal adenocarcinoma is seen to arise from the ductal epithelium accounting for over 90% of all pancreatic cancers. It arises most commonly (70%) in the head of the pancreas with infiltration into surrounding tissues including lymphatics, spleen, and the peritoneal cavity. Macroscopically, pancreatic cancers are firm, poorly defined tissue sclerotic masses with tongues of the cancer extending beyond the main tumour (Maitra and Hruban, 2008) (Vincent et al., 2011). It is characterised histologically by a dense stroma of fibroblasts and inflammatory cells, called desmoplasia which form a majority of the tumour mass with only a small number of cells being from the neoplastic ductal elements. The cancer primarily exhibits a glandular pattern with ductal structures demonstrating varying degrees of cellular atypia and differentiation. Vascular and perineural invasion is present in a majority of resected specimens along with metastasis to regional lymph nodes (Hruban, 2006; Vincent et al., 2011).

There are a number of histological variations of pancreatic cancer, which are less common, and have been enumerated in Table 1.2. It is important to differentiate these carcinoma owing to their better prognosis (colloid and medullary) in comparison to infiltrating ductal cancer (Hruban, 2006; Maitra and Hruban, 2008). The majority of pancreatic cancers express numerous immunohistochemically detectable proteins common amongst them being the carcinoembryonic antigen (CEA), carbohydrate antigen CA19-9, and CA-125. More recently members of the S100 family of calcium binding proteins S100A4, S100A6 and S100P have been identified and are now being utilised in

histological reporting of pancreatic cancer as they have prognostic value (Crnogorac-Jurcevic et al., 2003; Vimalachandran et al., 2005).

Malignant Tumours of the Exocrine Pancreas
1) Ductal adenocarcinoma (most common) 2) Mucinous noncystic carcinoma 3) Signet ring cell carcinoma 4) Adenosquamous carcinoma 5) Undifferentiated (anaplastic) adenocarcinoma 6) Undifferentiated carcinoma with osteoclast-like giant cells 7) Mixed ductal-endocrine carcinoma 8) Serous cyst adenocarcinoma 9) Mucinous cyst adenocarcinoma 1) non-invasive 2) invasive 10) Intraductal papillary mucinous carcinoma 1) non-invasive 2) invasive (papillary mucinous carcinoma) 11) Acinar cell carcinoma 1) Acinar cell cyst adenocarcinoma 2) Mixed acinar-endocrine carcinoma 12) Pancreatoblastoma 13) Solid-pseudopapillary carcinoma

Table 1.2: Malignant tumours of the pancreas

Pancreatic cancer precursor lesions

Clinical and histological studies have linked precursor lesions to pancreatic cancer. The most common and extensively studied lesion is pancreatic intraepithelial neoplasia, PanIN which is found in the smaller calibre pancreatic ducts (<5mm) (Hruban and Adsay, 2009; Hruban et al., 2001; Iacobuzio-Donahue, 2011). PanINs can be either papillary or flat being composed of cubodial cells with varying amount of mucin. Other precursor lesions analysed are mucinous cystic neoplasm (MCN) and Intraductal papillary mucinous neoplasm (IPMN) (Matthaei et al., 2011). IPMNs are similar to PanINs in their architectural structure however grow into large cystic lesions. MCNs are large mucin producing epithelial lesions with a cystic architecture harbouring a distinctive ovarian-type stroma. They arise in the main pancreatic duct or one of its ductal structures (Matthaei et al., 2011). Autopsy findings and analysis of resected pancreatic cancer specimens show the occurrence of PanINs alongside pancreatic adenocarcinoma, suggesting a biologic and developmental relationship (Hruban et al., 2001; Hruban et al., 2000).

PanINs lesions show a spectrum of varying morphology compared to ductal elements and are therefore graded from stages I to III (Hruban et al., 2000) (Hruban et al., 2008). The earliest stage is characterized by the appearance of a columnar, mucinous epithelium, with increasing architectural disorganization and nuclear atypia appearing through stages II and III. Invasion beyond the basement membrane signifies the transformation to frank pancreatic ductal adenocarcinoma (Hruban et al., 2001). Studies for molecular profiling have over the last two decades reinforced the PanIN to PDAC progression model through

documentation of an increasing number of gene alterations (Campbell et al., 2010; Hruban and Adsay, 2009; Iacobuzio-Donahue, 2011; Rhim et al., 2012). A standard classification system has been put forward by the National Cancer Institute (NCI) Cancer Think-tank in 2000 (Hruban et al., 2001) and is now used as standard both in research and for histological reporting worldwide.

Pancreatic Cancer Progression Model

The standard classification system put forward in 2000 (Hruban et al., 2001) was based on the fact that genetic mutations occurring in pancreatic cancer appear to be in a temporal, ordered sequence rather than a random fashion. This confirms the theory that neoplastic progression is a result of a combination of growth promoters and inhibitors. A pancreatic cancer progression model show below has been taken from Hezel *et al.* 2006, indicating the pancreatic cancer progression and critical genetic mutation (Figure 1.3).

KRAS mutations and telomere shortening are seen as early events, being demonstrable in the pancreatic cancer progression model and present in up to 87% of cancer associated PanIN 2/3 lesions (Scarlett et al., 2011) (Maitra et al., 2005). The high frequency of *KRAS* mutation detected in PanINs support its role as an initiating event in the development of invasive disease (Hingorani et al., 2003; Hruban and Adsay, 2009). Inactivation of $p16^{INK4A}$ gene has been detected in PanIN-1 lesions, however is more commonly seen in intermediate lesions (PanIN 2/3) with inactivation found to increase up

to 85% in invasive cancers (Scarlett et al., 2011). Late occurring genetic aberrations in the pancreatic cancer progression model are overexpression of *p53* and loss of *SMAD4* which are seen to occur in later stages in PanINs and adenocarcinoma, when the tumour has attained significant dysplasia (Maitra et al., 2003) (Luttges et al., 2001). Occurring in less than 50% of PanIN-3 lesions (Schwarte-Waldhoff et al., 2000) loss of *SMAD4* expression is predominately seen in invasive cancers.

The role of PanINs as preinvasive lesions which share many histological and molecular features with advanced carcinomas excepting breachment of the basement membrane has recently been challenged by Rhim *et al.* (Rhim et al., 2012). Contrary to prevailing concepts, the authors showed that low-grade PanINs that harbour *KRAS* mutations have migrated into the surrounding tissue in genetically engineered mice. Additionally, they showed that the preneoplastic cells which had migrated into the stroma exhibited features of mesenchymal cells. These features highlighted the long standing debate of epithelial to mesenchymal cell transition, changing fundamentally our concept of pancreatic cancer development and progression, in that very early precursor cells, until now thought to lack invasive properties, were capable of invasion and seeding distant sites (Rhim et al., 2012).

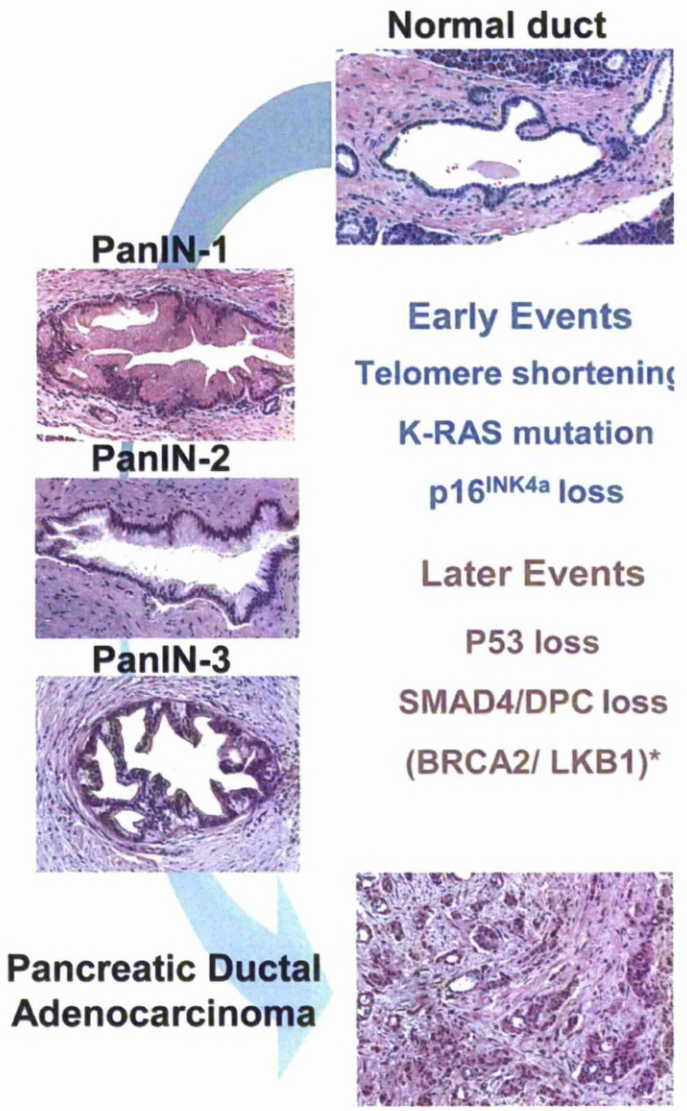


Figure 1.3: Pathological and molecular progression model of pancreatic cancer. Taken from Hezel, *et al.*, Genetics and biology of pancreatic ductal adenocarcinoma (Hezel et al., 2006)

CHAPTER TWO

THE CANCER MICROENVIRONMENT

2.1 The Tumour Microenvironment

The tumour microenvironment is composed of an ecosystem of tumours cells, resident and infiltrating non-tumour cells termed the 'stroma' along with molecules, which serve as chemomediators (Joyce and Pollard, 2009; Neesse et al., 2011; Shields et al., 2012b; Witz, 2008). A distinct molecular 'cross talk' between the host and its surrounding cells is seen to occur and is currently the subject of study in a variety of cancer settings (Polyak et al., 2009). The complex interplay, between parenchymal cells and their microenvironments is aimed at maintaining a homeostatic balance however, disruption of the interplay can induce aberrant cell proliferation, adhesion, function and migration. This process is considered to promote the initiation of malignancy with studies indicating that changes in stromal behaviour can promote epithelial transformation (Polyak et al., 2009).

Locally activated cellular and extracellular elements of the host microenvironment secrete molecules that can also influence the malignant phenotype and modify the proliferative and invasive behaviour of the tumour (Whiteside, 2008). This has been proposed to occur via increasing the genetic instability of tumour cells, inducing signalling cascades in tumour cells through tumour-associated receptors and by exerting selective pressures on tumour cells (Shapiro et al.) (Liotta and Kohn, 2001). A better understanding of stromal epithelial paracrine interaction pathway has brought forward many new concepts in tumour initiation, proliferation and opened the ways for new therapeutic targets (Polyak et al., 2009). The hedgehog signalling pathway in pancreatic cancer is one such example where cancer associated fibroblasts and not epithelial cells as

previously thought) are responsible for enhanced epithelial cell proliferation providing therapeutic targets (Yauch et al., 2008).

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells can convert to mesenchymal cells, being essential for organogenesis however, in cancer it can contribute to tumour cell heterogeneity and metastatic progression. Stromal and epithelial interactions in tumour microenvironment have an important role in the EMT process and the concepts of co-evolution of tumour cells and their microenvironment has met with some controversy (Sipos and Galamb, 2012) (Hill et al., 2005). Different putative models are proposed, some suggesting it is the stromal cells such as genetically altered fibroblast that can initiate tumourogensis in epithelial cells (Kuperwasser et al., 2004). In pancreatic cancer Rhim *et al.* recently published a paper suggesting that inflammation enhances cancer progression in part by facilitating EMT and treatment with the immunosuppressive agent dexamethasone abolished dissemination (Rhim et al., 2012). Stromal and tumour interaction are widely studied in breast and prostate cancers, with fibroblasts and inflammatory cells being at the centre of attention. Stromal fibroblasts in prostate cancer contribute towards the tumour's formation, with a similar effect exerted by fibroblasts in breast cancer progression. Macrophages similarly have a role in promotion of extravasation, seeding and persistent growth of breast cancer cells (Qian et al., 2011; Yates, 2011).

In contrast, the tumours seem to have a greater impact on its host stroma in terms of signalling and recruitments of cells. It is well established that the malignant tumour cells

recruit vasculature and stroma cells through secretion and production of stimulatory growth factors and chemokines (He et al., 2011) (Friedl and Alexander, 2011). This signalling can result in modification of the microenvironment by the tumour cells themselves, in order to facilitate tumour invasion and progression. Moreover, it has been revealed that stromal cells are also recruited to the microenvironment from the circulation and bone marrow to allowing growth of the primary cancer to facilitate metastatic dissemination (Gil-Bernabe et al., 2012; Hiratsuka et al., 2006; Joyce and Pollard, 2009). It is now recognised that the complicated interactions between tumour cells and surrounding stromal host cells is a vital regulator for conferring the metastatic potential of cancer cells.

The concept of the tumour and microenvironmental interactions is not new. In fact Stephen Paget put forward the concept of the 'seed and soil' about 100 years ago. He postulated that the microenvironmental components (the 'soil') exert a selective pressure on the tumour cells (the 'seed') which determines the cancer cell's malignant phenotype driving tumour progression and metastases (Witz and Levy-Nissenbaum, 2006). In addition, specific stromal components have also emerged as markers of poor survival in cancer patients (Bingle et al., 2002; Koukourakis et al., 2003; Neesse et al., 2011).

2.2 Pancreatic Cancer Microenvironment

There has been an extensive focus in genomic research to further elucidate our understanding of genetic alterations in pancreatic cancer cells (Jones et al., 2008), with attention now increasingly expanding towards the microenvironment, in particular the stromal cellular compartment (Lonardo et al., 2012) (Neesse et al., 2011). Pancreatic cancer, besides consisting of a collection of homogenous cancer cells, demonstrates a characteristically intense desmoplastic stroma which can account for up to 75% of the pancreatic tumour volume(Seymour et al., 1994) . The stroma consists of an extracellular matrix maintained by diffusible paracrine growth factors and cytokines, a cellular tissue framework of tumour associated vascular, inflammatory and fibroblastic cells (Friedl and Alexander, 2011). These components of the stroma are either locally derived or recruited from the circulation and surrounding interacting with the cancer cells (Chu et al., 2007; Farrow et al., 2008). Iacobuzio-Donahue *et al.* subdivided the pancreatic host stromal compartments into two distinct regions, juxtatumoral stroma and panstroma (Iacobuzio-Donahue et al., 2002b). The term 'juxtatumoral stroma' refers to stromal cells, which are in direct contact with the tumour, while 'panstroma' refers to all stromal cells within the desmoplastic response to the infiltrating tumour (Figure 1.4). The pattern of gene expression in these stromal sub-compartments was found to be distinct (Iacobuzio-Donahue et al., 2002b) potentially reflecting specific functions for each sub-compartment.

Pancreatic cancer cells have been shown to modify the extracellular matrix components through paracrine effects (Koninger et al., 2004). In healthy tissue, complex

communications between host cells maintains a healthy tissue homeostasis, inhibiting inappropriate mixing of cells from differing tissue types. Malignant cells, in contrast, ignore these signals and dominate the local host cell populations through the production of stimulatory growth factors and cytokines. Desmoplasia containing extracellular matrix proteins and cells can therefore modulate the growth of pancreatic cancer cells providing a scaffolding for cancer growth (Pandol et al., 2009). Histologically PDAC tumour consists of cancer cells and a stromal compartment which can vary significantly from tumour to tumour suggesting that stroma formation depends on a complex set of interactions between cancer cells, non-malignant cells and extracellular matrix (ECM) in a particular tissue(Chu et al., 2007). More recently, pancreatic cancer subtypes have been described following transcriptional profiles of cancer samples and cell lines. Further gene signatures for these subtypes have also been defined, which may have utility in stratifying patients for their response to different therapeutic agents (Collisson et al., 2011).

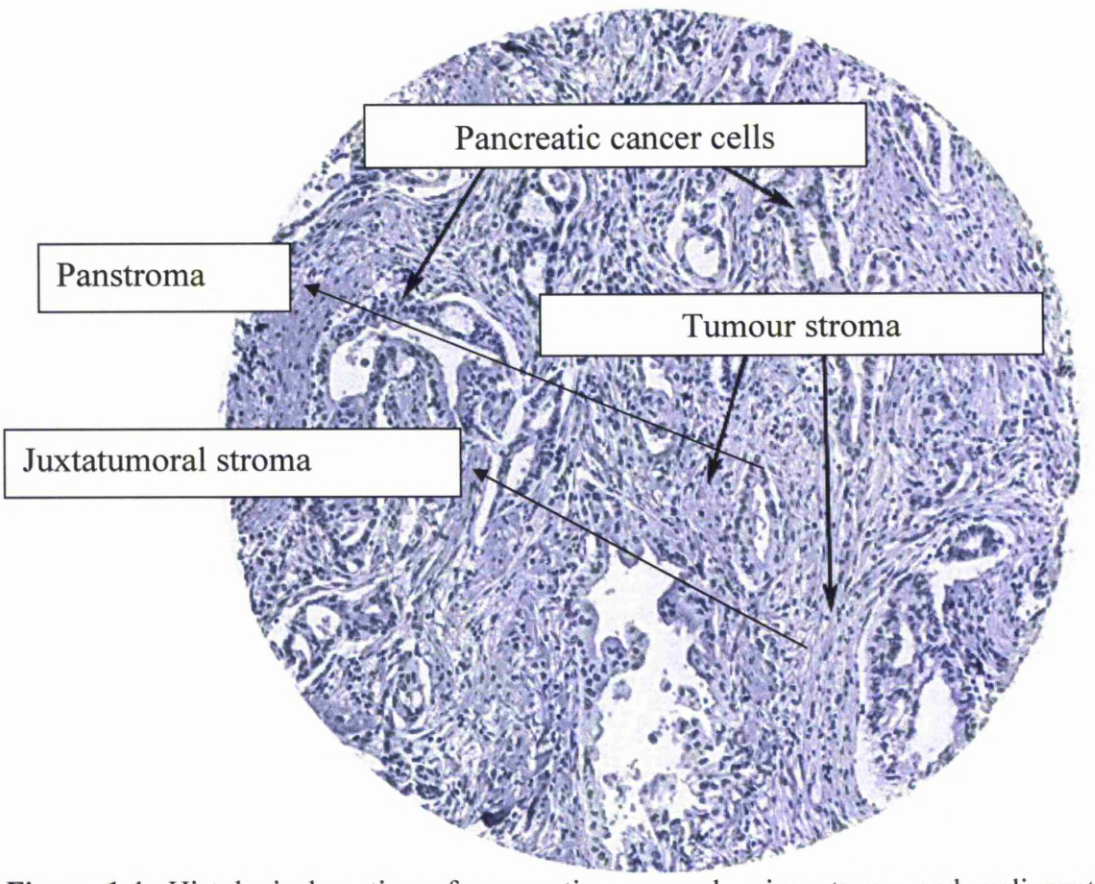


Figure 1.4: Histological section of pancreatic cancer showing stroma and malignant ductal epithelial cells

PanIN stroma formation

PanINs which represent precursor lesions which arise in the normal pancreatic tissue (Hruban et al., 2001). Being an in-situ neoplasm it has been described as confined to the basement membrane, however recent publications have challenged this concept suggesting that early preinvasive precursors were seen to invade and spread beyond the basement membrane (Rhim et al., 2012). The stroma surrounding the PanIN lesions

shows a variable response based on the aggressiveness of the lesion. PanIN associated stroma is characterised by neovascularisation and increased inflammatory cell count and it is therefore suggested that the stroma surrounding PanINs promote tumour growth, angiogenesis, ECM remodelling aiding tumour invasion converting an insitu lesion to an invasive one (Detlefsen et al., 2005) (Scarlett et al., 2011).

2.3 Components of pancreatic cancer stroma

The extensive stromal response seen in pancreatic cancer is a complex structure characterised by activation of pancreatic stellate cells (which have myofibroblastic properties), vascular proliferation, infiltration of inflammatory cells and extensive extracellular matrix formation (Farrow et al., 2008) (Neesse et al., 2011). The above responses can be divided into 3 broad areas, which have been discussed in subsequent sections in further detail. They are:

(1) **Extracellular matrix components** (the framework interacting with the cellular components exerting signalling properties)

(2) **Stromal cellular components**

(3) **Cell mediators** (including growth factors and cytokines)

Extracellular matrix components

The extracellular matrix (ECM) is part of the dense stromal response seen in pancreatic cancer and it is an integral part of the functional network of cell to cell interactions contributing to shaping the metastatic character of pancreatic cancer (Kresse and Schonherr, 2001). There is a multiplicity of matrix proteins present in pancreatic cancer primarily formed by collagens types I and IV (Shields et al., 2012b). Additionally, adhesive glycoprotein such as laminin, fibronectin, vitronectin and tenascin are present in the pancreatic cancer microenvironment (Bachem et al., 2005) which exert signalling properties either through interactions with growth factors or directly (Schonherr et al., 2001). Koninger *et al.* have shown that small leucine rich proteoglycans (SLRPs), decorin and lumican are present in abundance in the pancreatic cancer microenvironment and are now known to be produced primarily by the stellate cells rather than the primary pancreatic carcinoma itself (Koninger et al., 2004).

The fibrotic matrix was initially regarded as a host barrier against cancer invasion however, it is now evident that it can modulate and initiate carcinogenesis by influencing growth, differentiation and motility of cancer (Bissell and Radisky, 2001). The extracellular matrix undergoes a constant remodelling process primarily by proteases, most notably the metalloproteinases which aid in the regulation of a wide variety of cellular processes from proliferation to cell death therefore inducing tumour progression (Stamenkovic I, 2003) (Munshi and Stack, 2006; Pupa SM et al., 2002). The fibrillary collagens, fibronectin and laminin increase cancer cell growth and promote resistance to chemotherapeutic agents in a variety of pancreatic cancer cell lines. Type I collagen can

regulate cellular proliferation modulating intracellular signal transduction affecting cell-cycle regulatory proteins cyclins and CDKs (Van Hoorde et al., 2000).

Cell migration is an essential part of tumour invasion with the loss of cellular adhesions being a key event in tumour invasion and progression. There are a number of glycoprotein adhesion receptors that mediate cellular adhesion, an important one being integrin. These transmembrane proteins combine with the extracellular matrix components and to the intracellular actin cytoskeleton. The integrins are also known for activation of intracellular signal transduction pathways (Danen EHJ and Sonnenberg A, 2003). Specific integrins act as receptors for structural ECM proteins such as laminin and fibronectin allowing for signalling and adhesion (Heino J, 1996). Increased expression of laminin-binding integrins or decreased expression of fibronectin integrins has been associated with aggressive growth and metastatic capacity in pancreatic cancer (Arao S et al., 2000). Fibrinogen has been shown to be induce the production of interleukin 6 (IL6), interleukin 8 (IL8), monocyte chemoattractant protein-1, vascular endothelial growth factor, and type I collagen in pancreatic stellate cells with integrins working as counter receptors for fibrinogen in these cells demonstrating molecular interactions in the pancreatic cancer stroma (Masamune et al., 2009a).

Matrix Metalloproteinase

During the invasive process cancer cells rely on activation of the surrounding stroma, resulting in disruption of the periglandular basement membrane and hemi-desmosome

structure (Shields et al., 2012b). Stromal and tumour cell interactions produce modifications to the adjacent ECM allowing for favourable grounds to allow for microinvasion (Hwang et al., 2008). Enzymes that degrade the ECM and its associated proteins are tightly regulated and are produced both by tumour cells and host stromal cells. These enzymes can be classified into two categories (1) Matrix metalloproteinase (MMPs) and (2) Tissue serine proteases

The Matrix metalloproteinase family have an important regulatory function in maintaining the extracellular environment aiding in the remodelling and hence invasion of pancreatic cancer cells. MMPs are expressed in pancreatic cancer cells, fibroblasts, activated pancreatic stellate cells and immunocytes, with MMP 1,2,3,7,9,11,13 been described in pancreatic ductal cancer cells (Duner et al., 2010; Kordes et al., 2005). The MMPs are a family of highly conserved zinc dependant endopeptidases, which collectively are capable of degradation of the basement membrane, in particular collagen type IV (Phillips PA et al., 2003). MMPs, can be subdivided into four groups: the collagenases, the stromelysins, the gelatinases and the membrane type MMPs (MT-MMPs) (Munshi and Stack, 2006). Several of the MMPs have been localised to the same chromosomal location, 11q23, a region which is amplified in several solid organ human tumours (Rooney PH et al., 1999).

Of note in pancreatic cancer are MMP-2 and MMP-9, with the latter having the ability to mobilise VEGF hence playing a vital part in angiogenesis (Bergers G et al., 2000; Harvey SH et al., 2003). Pancreatic stellate cells and macrophages predominantly secrete

gelatinases (MMP2, MMP9), which degrade the basement membrane collagen (type IV) and are associated with inflammation, fibrosis, angiogenesis, and cancer invasion (Duner et al., 2010; Phillips et al., 2003). MMP-7 is present in most PDACs, in particular in the epithelial cells at the invasive tumour front and is significantly correlated with advanced pathologic stages, and poor prognosis (Yamamoto et al., 2001). MMP11 is expressed in pancreatic cancer stromal cells in a juxtatumoural distribution and may indicate a direct communication between tumour and stromal cells (Iacobuzio-Donahue et al., 2002b).

The activities of MMPs can be blocked by non-specific inhibitors such as α2-macroglobulin and α1-antiprotease, the main physiological inhibitors are the tissue inhibitors of metalloproteinase (TIMPs) (Curran S and Murray GI, 1999). Several different TIMPs have been identified (TIMP 1- 4) and studies have found an inverse relationship between TIMP-1 expression and metastatic potential (Bloomston M et al., 2002) (Alexander CM and Werb Z, 1992). Activation of pro-MMP2 to MMP2 by membrane bound MT-MMP is a critical step in pancreatic cancer (Bramhall SR et al., 1997) with pro-MMP2 being produced by stromal cells and activated by pancreatic cancer cell membrane bound MT-MMP. Similarly, MT1-MMP is overexpressed in pancreatic cancer cells at sites of PDAC invasion (Iacobuzio-Donahue et al., 2002b) with genetic studies indicating MT1-MMP to be a primary regulator of collagenolysis (Shields et al., 2012a). It has also recently been shown to have a role in generation of collagen rich stromal reaction with mouse models demonstrating that high MT1-MMP presence is associated with large stromal dense pancreatic cancers having increased TGF-β1

signalling. This suggests that MMPs are vital mediators at the cross roads of pancreatic cancer and stromal interactions having a role in cancer progression (Krantz et al., 2011).

Serine proteases

The urokinase-type plasminogen activation (uPA) system is a family of serine proteases, primarily focused at proteolytic degradation involved in thrombolysis and extracellular matrix degradation (Berger DH, 2002). Invading tumour cells have proteolytic activity on their cell surface mediated through receptors for the serine proteases. uPA released from either the tumour or stromal cells, bind to these tumour cell receptors (uPA-R) which focuses the enzymatic proteolytic activity to the tumour cell surface. Urokinase Plasminogen activator (uPA) converts inactive plasminogen to plasmin, which acts both directly and indirectly (through the activation of MMPs) to degrade extracellular matrix proteins and thereby facilitate cell proliferation, invasion and metastasis (Andreasen PA et al., 1997).

Binding of uPA to its receptor also initiates intracellular transduction eliciting a variety of cellular functions such as migration of endothelial cells, chemotaxis of neutrophils and tyrosine phosphorylation (Nguyen DHD et al., 2000). Experimental models have indicated an association of uPA in the development of metastases with high levels of uPA appearing to predict a poor prognosis in human cancers (Kim SJ et al., 1998). In pancreatic cancer specifically, activation of the uPA system mediates the invasiveness of

pancreatic cancer cell lines, and tumour cell motility can be inhibited by uPA inhibitors (Diaz VM et al., 2002).

Stromal cellular component

The stroma is composed of a number of different host cell types, which are both locally derived and/ or recruited from the circulation (Joyce and Pollard, 2009). Even though there is structural heterogeneity, tumour stroma can be broken down into constituent parts.

Mesenchymal cells

These cells represent a population of spindle shaped cells comprising mainly of traditional fibroblasts, which normally form the bulk of the connective tissues support. These fibroblasts are present around the ducts, interlobar spaces and blood vessels forming an essential part of the microenvironment exhibiting proliferation in pancreatic injury such as pancreatitis and PDAC (Hwang et al., 2008). Other mesenchymal cells include pericytes that are derived from bone marrow (Iacobuzio-Donahue et al., 2002a). The mesenchymal cell population is composed of a variety of cell types, which are constantly believed to be interacting with the surrounding tumour. Molecular analysis of pancreatic cancer tissue has suggested patterns of stromal heterogeneity with invasion associated genes found to be expressed differentially in the 2 stromal compartments, the juxtastroma and the panstroma (Iacobuzio-Donahue et al., 2002b).

Fibroblasts upon stimulation may proliferate to produce an extracellular matrix rich in collagen. Embedded within the extracellular matrix niche they characteristically express vimentin and fibroblast specific protein-1 (FSP1) (Kalluri and Zeisberg, 2006). Important functions, other than production of extracellular matrix, include epithelial differentiation, regulation of inflammation and involvement in wound healing by synthesising collagen type I, II and IV (Chu et al., 2007; Neesse et al., 2011). Basement membrane production and remodelling through protease metalloproteinases (MMPs) are crucial functions of fibroblasts in maintaining the ECM homeostasis (Neesse et al., 2011). In the cancer setting fibroblasts can acquire an activated phenotype, triggered by growth factors such as transforming growth factor beta (TGF-β), epidermal growth factors (EDGF), platelet derived growth factor (PDGF), and fibroblast derived growth factors (FGF) which are released from cancer epithelial cells, infiltrating mononuclear cells and macrophages (Mahadevan and Von Hoff, 2007). Fibroblasts can also be activated by direct cell-to-cell communication via intercellular adhesion molecules (ICAM) or vascular adhesion molecules (VCAM1) (Clayton et al., 1998).

Activated fibroblasts express smooth muscle actin (α-SMA) converting them into myofibroblasts (Bachem MG et al., 1998) which allow them to develop contractile characteristics, often being termed "carcinoma induced fibroblasts" (Chu et al., 2007). These cells have the capacity to express growth factors such as vascular endothelial growth factor, insulin like growth factor and hepatocyte growth factor (Neesse et al., 2011). Furthermore, they also express ECM degrading metalloproteinases MMP2, MMP7 and MMP9 which facilitate ECM turnover (Hwang et al., 2008). In pancreatic cancer

there is accumulating evidence to suggest that cancer-associated stromal fibroblasts (CAF) contribute to tumour growth and the desmoplastic reaction by actively communicating with cancer cells, myofibroblasts and pancreatic stellate cells (Lonardo et al., 2012; Walter et al., 2010). In other cancers, i.e. breast adenocarcinoma, CAFs have been demonstrated to aid angiogenesis (Kitamura et al., 2007). Gene expression profiling of human pancreatic CAFs and nonneoplastic pancreatic fibroblasts have identified the hedgehog receptor smoothened (SMO) to be upregulated in CAFs relative to control fibroblasts suggesting that stromal cells may be a therapeutic target for smoothened antagonists in pancreatic cancer (Walter et al., 2010).

The activated cancer associated fibroblasts are not a homogenous entity but are a collection of cells with a varying genotype reflected in their differing cellular functioning cytokine and ECM production and paracrine effects (Kalluri and Zeisberg, 2006). They remain an area of active interest amongst scientist owing to their ever complex role and interactions.

Pancreatic stellate cells (PSC)

Pancreatic stellate cells are a group of quiescent state cells, which comprise approximately 4% of the pancreatic cell population and are a major contributor towards stromal formation in both cancer and the pancreatitis setting (Masamune et al., 2009b) (Duner et al., 2010; Lonardo et al., 2012). Their origins are uncertain and they may be derived from circulating progenitor cells being identified by their spindle shaped

appearance as they lie in the periacinar area. They express desmin and glial fibrillary acidic protein (Farrow et al., 2008). They also have the ability to store vitamin A which makes them similar to hepatic stellate cell (Duner et al., 2010). Upon stresses due to pancreatic injury or inflammation, stellate cells become active in a similar fashion to myofibroblasts like cells, expressing α-SMA (Bachem MG et al., 1998; Kleeff et al., 2007). Studies reveal that soluble cytokines (interleukin IL-1, IL-6), tumour necrosis factor alpha (TNF-α) and growth factors e.g. platelet derived growth factor (PDGF), TGF-β1, and fibroblast growth factor-2 are regulators of PSC activation (Masamune and Shimosegawa, 2009) (Bachem et al., 2005) (Duner et al., 2010). These are produced by a host of cells in the microenvironment, such as acinar cells, inflammatory cells, ductal cells, endothelial cells and cancer cells.

Myofibroblasts and pancreatic stellate cells have a wide range of functions, including organogenesis (Simon-Assmann P et al., 1995), wound healing (Moore R et al., 1998), angiogenesis, production of cytokines, growth factors (IL-1β, IL-6, TNF-α, TGF-β1) ECM proteins and metalloproteinases (MMP-2, MMP-9, MMP-13) , as well as inhibitors of MMPs (Masamune et al., 2009a; Vonlaufen et al., 2008) (Kleeff et al., 2007) (Hwang et al., 2008). Stellate cells also produce chemokines such as monocytes chemoattractants and IL-8, which aid recruitment of inflammatory cells to the pancreatic cancer microenvironment (Andoh et al., 2000). These cytokines produced by stellate cells have a proliferative effects on pancreatic cancer cells in experimental models with these cells exhibiting increased Notch signalling (Fujita et al., 2009).

More recently, PSCs have been identified to express toll-like receptors which are involved in activation of innate immunity (Masamune et al., 2008b). They also have a role in phagocytosis displaying a "macrophage" like function comparable to Kupffer cells in the liver, playing a part in maintaining homeostasis of the pancreas (Masamune et al., 2009b). PSCs are proangiogenic, expressing vascular endothelial growth factor as well as for VEGF and angiopoietin-1 receptors. In vitro conditioned media from stellate cells is seen to induce angiogenesis (Masamune et al., 2008a).

Pancreatic stellate cells have emerged as one of the major mediators of the increased integrins mediated cell-to-cell adhesion, and migration of pancreatic cancer cells (Fujita et al., 2009). Lonardo *et al.* have also very recently demonstrated that PSCs are important in forming a paracrine niche for pancreatic cancer stem cells promoting their invasiveness and highlighting a target for therapeutic intervention (Lonardo et al., 2012). Similarly, SPARC (secreted protein acidic and rich in cysteine) is also expressed in high quantities in pancreatic stellate cells promoting PDAC cell invasiveness and poor prognosis (Chen et al., 2010). Strikingly PSCs have also been demonstrated in metastatic foci of nude mice suggesting a co-migration to produce favourable microenvironment conditions at distant sites of metastases (Vonlaufen et al., 2008).

Immune cells

Immune cells comprise of a variety of different cells (lymphocytes, dendritic cells, neutrophils, macrophages, monocytes, mast cells, natural killer cells and eosinophils).

Together they make up the adaptive and innate immunity of the body. Their morphological appearance is similar to granulation tissue and desmoplasia (Coussens and Werb, 2002), though their role in pancreatic cancer progression is a matter of investigation (Ang et al., 2010). There tends to be less immune cell infiltrate around the tumour glands with clustering of immune cell around the invasive tumour front (Pandol et al., 2009). Stroma in pancreatic cancer is seen to have an increased numbers of T-cells, mast cells and macrophages as well as monocytes (Emmrich et al., 1998) (Sheikh et al., 2007; Wachsmann et al., 2012) and these may participate in cancer cell growth either directly or indirectly by altering the tumour microenvironment (Ang et al., 2010; Whiteside, 2008).

Pancreatic cancer cells in contrast to other cancers express a variety of cancer associated antigens that can potentially be recognized by T-cells and publications have demonstrated that there is a surge in functionally competent $CD4^+$ and $CD8^+$ T cells in the bone marrow and peripheral circulation of these patients (Kubuschok et al., 2004) (Schmitz-Winnenthal et al., 2005). T-cells rely on dendritic cells for antigen presentation and hence stimulation. Dendritic cells once stimulated allow for activation of tissue and bone marrow based T-cells. Dendritic cell stimulation is modulated by a variety of signals such as heat shock proteins, ECM degradation products and suppressed by cytokines such as interleukin-10 (IL-10), TGF-β1 with the latter (suppressive cytokines) being produced in abundance by pancreatic stellate cells and infiltrating macrophages (Esposito et al., 2004). As a result of this, pancreatic cancer, through stromal activation can induce an immune suppressive microenvironment. In the tumour mass itself, pancreatic cancer is

characterized by the presence of few tumour-specific $CD8^+$ T cells, B-cells, and tumour-reactive antibody-producing plasma cells. Moreover, when evaluated the presence of both $CD4^+$ and $CD8^+$ T-cells in malignant PDAC tissue correlates with a better prognosis (Fukunaga et al., 2004). Pancreatic cancer can has been shown to successfully employ various mechanisms to evade immune surveillance. These involve down regulation of major histocompatibility complex molecules, rendering neoplastic cells more resistant to recognition by T-cells (Wachsmann et al., 2012), secretion of inhibitory cytokines such as IL-10 and TGF-β1 which further reduce local T-cell activity (Wachsmann et al., 2012).

Natural killer cells are a subset of cytotoxic lymphocytes that only recently received attention for their role in tumour development and progression (Wachsmann et al., 2012). Natural killer cells do not express unique antigen-specific receptors, but they play an important role in innate immunity and antitumor immunity (Raulet and Guerra, 2009). In PDAC, two phenotypically and functionally distinct subsets of NK cells are present based on the levels of CD56 expression and cytokine secretion. Natural killer cells in PDAC have been reported to mediate tumour cell lysis with the presence of high levels of these cells conferring a better prognosis (Degrate et al., 2009).

There are few studies involving the role of mast cells in PDAC, however studies on mast cells and macrophages have indicated that these cells express angiogenic factors (VEGF) aiding in tumour neovascularisation (Esposito et al., 2004). Strouch *et al.* has demonstrated that mast cell infiltration was significantly increased in pancreatic cancer

compared with normal controls and correlated with higher-grade tumours, decreased recurrence-free and disease-specific survival (Strouch et al., 2010).

Vascular tissue

Angiogenesis is essential for tumour growth and progression, which is why not only pancreatic cancer but all solid tumours undergo a process of dynamic neovascularisation and vascular remodelling (Chu et al., 2007). Vascular tissue formation is to a greater extent controlled by the balance of pro- and anti-angiogenic factors produced by a variety of cancer cells and stromal cells with vascular endothelial growth factor-A (VEGF-A) at the centre of this function. This initiation of new vessel formation, the so called "angiogenic switch", occurs very early on in tumour development and is essential for tumour development (Ribatti et al., 2007; Ruoslahti E, 2002). Early tumour angiogenesis may be due to the hypoxic environment. Pericytes, which are mesenchymal cells, defined by their close association with endothelial cells have been implicated as vital regulators of angiogenesis including vascular development, stabilization, and remodelling (Armulik et al., 2005). Pericytes are fairly plastic cells and can differentiate into vascular smooth muscles as well as stromal cells (Armulik et al., 2005). Pancreatic cancer angiogenesis is not controlled by the expression of VEGF-A in the tumour and stromal cells alone, but is a complex balance of other signalling molecules such as metalloproteinases and platelet derived growth factors (PDGF) which are present in the microenvironment (Korc, 2003; Song et al., 2005). The immune cells, which contribute towards the inflammatory response in the tumour microenvironment, also have a crucial role in vascular tissue

development. Infiltrating immune cells mainly neutrophils and macrophages are proangiogenic conferring a worse prognosis (Imhof and Aurrand-Lions, 2006) (Tartour et al., 2011). Although PDAC is not a vascular tumour, it has areas of enhanced endothelial cell proliferation with significant correlations between blood vessel density and disease progression, suggesting that antiangiogenic therapy many have a role as a therapeutic target. Moreover, although antiangiogenic strategies that target VEGF alone have not yet shown efficacy for the treatment of pancreatic cancer, some antiangiogenic molecules reduce the immunosuppression associated with cancer (Tartour et al., 2011
) by decreasing immunosuppressive cells (T-cells) and immunosuppressive cytokines (IL-10 and TGF-β). This suggests the links in the pancreatic cancer microenvironment between cells of immune system and vascular tissue engaged in cross-talk.

Cytokines expression in the pancreatic cancer microenvironment

Cytokine production into the local microenvironment of the pancreas plays a important role in the basis of molecular cross-talk between epithelial derived tumour cells and its surrounding mesenchymal stromal cells (Farrow and Evers, 2002; Kleeff et al., 2007) (Bellone et al., 2006; Farrow et al., 2008). These soluble messengers act in a paracrine and / or autocrine fashion, and are responsible for a variety of biological processes including cell proliferation, modulation of host immune response, tumour growth, cell signalling, cell trafficking, neovascularisation and metastasis. Cytokines have a varying function most importantly working in combination with others mediators maintaining a

homeostatic balance. Using serum from pancreatic cancers patients and pancreatic cancer cell lines, Bellone *et al.* evaluated the cytokine profile in the tumour microenvironment (Bellone et al., 2006).

Roles of cytokines in tumour biology vary, some have been termed as "protective" with moderate expression, appearing to confer some survival advantage whereas elevated levels of expression of others correlate to poor survival. Sources of cytokines once again vary and are produced by the pancreatic cancer cells or being expressed by the stromal cells (Ang et al., ; Sheikh et al., 2007). Salient cytokines present in the microenvironment of pancreatic cancer are detailed below.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is a major inducer of tumour vascularisation which is essential for tumour sustainability (Ferrara N and Davis-Smyth T, 1997). In situ hybridisation studies have confirmed that VEGF mRNA is up regulated in many human tumours (Ferrara N and Keyt B, 1997). Studies in pancreatic cancer have revealed that overexpression of VEGF correlates with microvascular density, tumour progression and poor prognosis (Wachsmann et al., 2012) (Kuwahara K et al., 2003).

Soluble VEGF binds to receptors of VEGF (VEGFR1/ VEGFR2) which are also overexpressed in pancreatic cancer (Itakura et al., 2000). VEGF/VEGFR-2 complexes on

endothelial cells can result in several downstream events that promote angiogenesis. Both VEGF and VEGFR signalling is essential for growth of pancreatic tumours as demonstrated by inhibition leading to profound reduction in local and metastatic growth (Fukasawa and Korc, 2004; Ribatti et al., 2007). Trials using monoclonal antibodies to VEGF (bevacizumab) however did not yield a survival advantage compared to patients having standard chemotherapy (Van Cutsem et al., 2009).

Interestingly, angiogenic receptors may not only be expressed on endothelium but are also present on functional pancreatic cells. There is mounting evidence to suggest that high expression of VEGF is associated with poor prognosis and liver metastasis (Seo et al., 2000). It is therefore conceivable that agents targeting VEGF or its receptors may have a dual inhibitory effect on pancreatic cancer growth suppressing both angiogenesis and cancer cell function (Kleeff et al., 2007). Phase II trials utilising axitinib which is a VEGFR-1-3 and gemcitabine have also met with a discouraging result (Spano et al., 2008).

Hypoxia is seen to activate HIF-1 induction that can induce VEGF expression (Buchler P et al., 2003; Masamune et al., 2008a). In order to confirm that hypoxia activates HIF-1 induced VEGF expression, Buchler *et al.* analysed the mRNA expression of HIF-1 and VEGF in vivo. Low oxygen tension induced both HIF-1 and VEGF production in human pancreatic tissue and in cancer cell lines (Buchler P et al., 2003).

VEGF is not only expressed in the pancreatic cancer cell but is also shed into the microenvironment as a significant regulatory cytokine. Its production is also seen to occur in the stromal inflammatory infiltrate (mast cells and macrophages), which are recruited from the blood stream (Dineen et al., 2008). Angiogenesis is an important process in carcinogenesis originating from pre-existing venules within the tumour mass or the host tissue (Shannon AM et al., 2003). Tumour vessels, unlike normal vessels, are not arranged in a hierarchical fashion but haphazardly and are structurally heterogeneous and are hyper-permeable to plasma and proteins, as they are lined by actively dividing endothelial cells (Dvorak HF, 2003). Hyper-permeability is primarily a result of VEGF, which is 50,000 times more potent than histamine and hence important in the development of both tumour vasculature and tumour stromal elements (Dvorak HF, 2003). The mechanisms by which it does this is essentially the same as wound healing. As a result of vascular hyperpermeability and leakage of plasma, extravasated fibrin deposition in the tissue occurs which leads to formation of a fibrin gel, which is degraded and replaced by desmoplasia in the case of tumours or scar tissue in the case of wounds. VEGF is an essential modulator of this process and hence an anti-angiogenic approach to tumour treatment remains an important target for treatment particularly of advanced pancreatic cancer (Kleeff et al., 2007).

Interleukins

Interleukins are a group of communicatory cytokines first observed to be expressed in leukocytes (Sims et al., 1988) and since found to be produced by a wide variety of cells

including cancer cells. The majority of interleukins are synthesized by lymphocytes, as well as monocytes, macrophages, and endothelial cells.

Pancreatic cancer secretes and expresses a host of both proinflammatory (IL-1, IL-2, IL-6, IL-8, IL-12, and IL-18) and anti-inflammatory interleukins (IL-10, IL-11, IL-13) (Bellone et al., 2006). These interleukins are involved in a variety of cellular processes, both involving the stromal cell as well as the tumour cells, which include cellular proliferation, migration, metastasis, dampening and upregulating of immune response and angiogenesis (Feurino et al., 2006). IL-8 is a proinflammatory interleukin upregulated significantly in the pancreatic cancer microenvironment,(Farrow et al., 2008) with over 60% of resected cancer specimens displaying positive receptor expression (Kuwada et al., 2003). IL-8 also interacts closely with nitric oxide molecule, which is a regulator of vasodilation and angiogenesis in pancreatic cancer. High levels of nitric oxide results in the higher levels of IL-8 production and vice versa (Xiong, 2004).

Interleukins also serve as growth modulators with some increasing and others exerting a negative growth effect. IL-8 and IL-4 act as growth factors for pancreatic cancer as when inhibited in experimental conditions a reduction in cancer cell growth is demonstrated (Prokopchuk et al., 2005). Moreover, levels of IL-8 correlate with the metastatic potential of pancreatic cancer cell lines (Matsuo et al., 2004) as cells when cultured with IL-8 show an increase in cell growth and motility that is decreased when monoclonal antibodies to IL-8 are used (Matsuo et al., 2004). The presence of certain interleukins in the pancreatic cancer (IL-6 and IL-10) are a poor prognostic indicator and directly associated with poor

survival and recovery from surgery (Friess et al., 1999). Interleukins are important mediators of cell-to-cell interactions contributing towards the aggressive nature of pancreatic cancer.

Interleukins form communication channels between pancreatic cancer cells and host immune cells, which allow them to evade natural immune defence mechanisms (Ebrahimi et al., 2004). IL-10 is known to suppress T-cell activity in the pancreatic cancer microenvironment hence hindering anti-cancer response (Ebrahimi et al., 2004). Antigen presenting cells (Dendritic cells) in experimental conditions are inhibited by IL-6 produced by BxPC-3 pancreatic cancer cell lines (Feurino et al., 2006). IL-4 has a growth promoter effect on experimental cancer cell lines and mouse models alike (Kimura et al., 1999) with IL-12 in the other hand showed cancer growth inhibition in nude mice (Motoi et al., 2000). More recently, IL-6 and IL-8 have been shown in experimental conditions to induce and maintain EMT in pancreatic cancer cells when exposed to these soluble factor interleukins. IL-8 also has also shown to have an essential role for the acquisition and/or maintenance of the mesenchymal and invasive features in different cancer cells (Fernando et al., 2011; Huang et al., 2011).

Tumour necrosis factor

Tumour necrosis factor (TNFα and TNFβ) are monocytes/macrophage derived cytokines which are involved in tumour carcinogenesis, septic shock and systemic inflammatory response (Baran et al., 2009). Their primary role is regulation of immune cells with

dysregulation of production seen in a variety of human cancers (Locksley et al., 2001). In the pancreatic cancer setting, TNFα induces an increase in cell proliferation, which contrasts to the cytokine's inherent ability for tumour suppression. This is because TNFα has two receptors, RI that activates apoptosis, and RII which acts to increase levels of other growth factors such as epithelial derived growth factor and transforming growth factor alpha (TGF-α), resulting in enhanced cell growth (Schmiegel et al., 1993). Experimental evidence indicates that TNF-α interacts with other cytokines such as IL-8 affecting its induction in PANC-1, MIA PaCa-2 and BxPC-3 cancer cell lines(Takaya et al., 2000). TNF-α also aids the induction of monocyte chemotactic protein (MCP-1) which is a mediator for attracting monocytes, T-cells and other leukocytes (Takaya et al., 2000). This can possibly explain high levels of monocytic infiltration at varying degrees of maturation seen in the pancreatic cancer stroma (Sheikh et al., 2007).

One of TNF-α key functions is induction of apoptosis in cancer cells and recruitment of inflammatory cells in acute inflammatory conditions such as acinar injury (Farrow and Evers, 2002). In pancreatic cancer, TNF-α inhibits apoptosis thorough a mechanism centering on the NF-κB pathway. More recently its has been demonstrated that exogenous TNFα induced pancreatic cancer cell motility as well as epithelial-mesenchymal transition (EMT) (Baran et al., 2009). Functionally, however, TNF-α may cause greater tumour necrosis by increasing local thrombosis within the tumour vasculature leading to tumour shrinkage. It is this feature that can potentially be exploited for therapeutic benefit particularly in delivering radiation therapy (Feurino et al., 2006).

Growth Factors

Over expression of growth factors and their receptors is vital in the pathogenesis of pancreatic cancer. Growth factors act on nuclear transcription factors altering the expression of genes responsible for cell proliferation. They can also trigger cell death by activating specific cell surface receptors involved in cellular processes such as apoptosis. Some of these growth factors are produced by the cells of the tumour microenvironment highlighting the critical position tumour-host interactions occupy in carcinogenesis (Dranoff G, 2004).

Fibroblast growth factor (FGF)

The fibroblast growth factor family are a family of heparin binding polypeptide growth factors that are involved in mitogenesis, cell differentiation and angiogenesis (Ozawa et al., 2001). The FGF system has been implicated in the regulation of E-cadherin, an important adhesion molecule that is often altered in pancreatic cancer (El-Hariry I et al., 2001). Expression of FGF in pancreatic cancer is evident in both tumour as well as stroma cells, with signalling mediated by a dual receptor system comprising four high affinity transmembrane tyrosine-kinase FGF receptors (FGFR). This is mediated by a number of different intracellular pathways. Pancreatic stellate cells are known to produce FGF in response to different stimuli and have a role in maintaining homeostasis in the pancreatic cancer microenvironment (Duner et al., 2010). In pancreatic cancer, overexpression of FGF-1, FGF-2, FGF-5 and FGF-7 has been demonstrated (Kornmann M et al., 1998) with FGF-7 expression appearing to occur predominantly in the tumour

cells, as opposed to FGF-5 which localises predominantly to the stromal fibroblasts (Kornmann M et al., 1997). FGF-1, FGF-2 and FGF-5 enhance the growth of pancreatic cancer cells (Kornmann M et al., 1998) (Kleef J et al., 1998; Kornmann M et al., 1997). Over expression of FGFRs have also been demonstrated in pancreatic cancer (Ishiwata et al., 1998) (Kobrin MS et al., 1993), and may occur independently of FGF expression (Leung HY et al., 1994) suggesting they have an independent role involved in other pathways. Experimental evidence has suggested that conditioned media from pancreatic cancer cells stimulate pancreatic stellate cell activation via FGFs, the effects being neutralised by antibodies (Bachem et al., 2005) indicating their role in tumour cross talk. Furthermore, FGFRs signalling may contribute towards the desmoplasia as increased expression is seen in tumours with high levels of expression (Hezel et al., 2006).

Epidermal growth factor (EGF)

Epidermal growth factor (EGF) primarily functions in induction of cellular proliferation. Other members of its family include transforming growth factor alpha (TGF-α), and heparin binding EGF (HB-EGF). Its receptor is known as human EGF receptor-1 (HER1) and is closely related to several other similar receptors, including, HER-2 (c-erbB2), HER-3 (c-erbB3) and HER-4 (c-erbB4) (Ozawa et al., 2001).

Ligand binding results in phosphorylation of the receptors and through a series of intracellular signalling mechanisms involving tyrosine kinase, biological response ensues. These pathways culminate in an activation cascade involving RAF and MAP kinase which is a cellular process involved in cellular proliferation (Ozawa et al., 2001) (Seger

and Krebs, 1995; Tjomsland et al., 2011). Epidermal growth factor receptor overexpression may be detected in up to 90% of pancreatic tumours with high levels of EGF expression being associated with decreased survival and metastasis (Troiani et al., 2012). EGF has important paracrine interactions in the pancreatic cancer microenvironment as high levels are expressed in stromal cells with pancreatic cancer cells expressing receptors (Farrow et al., 2008; Tjomsland et al., 2011). EGF and its associated ligands have a part in the development of pancreatic cancer in transgenic mouse models with the transformed cells displaying an increased expression of EGF receptors and of the EGF itself. Two pharmacological agents, neutralizing monoclonal antibodies and small molecule tyrosine inhibitors have been employed to inhibit epidermal growth factor receptor function in pancreatic cancer treatment, however they have not been successful.

Crosstalk in the pancreatic microenvironment

Crosstalk between PDAC tumour cells and stromal components are complex interactions. Knowledge on this subject is still limited with some of these intricate pathways yet to be unravelled(Shields et al., 2012b). Cancer cells secret numerous growth factors such as TGF, VEGF, as well as ECM modifying matrix metalloproteinase, which all work towards stimulating angiogenesis, fibroblastic proliferation and recruitment of inflammatory cells (Matsuo et al., 2012; Neesse et al., 2011; Shields et al., 2012b). In a paracrine feedback loop, the affected cells stimulate proliferation of not only stromal compartment cells but also aid in the growth and proliferation of tumour cells.

CHAPTER THREE

SMAD4 AND TGF-β SIGNALLING IN PANCREATIC CANCER

3.1 *SMAD4*

A signature genetic mutation occurring frequently in PDAC is loss of the *SMAD4* (DPC4) transcriptional regulator (Matthaios et al., 2011; Mihaljevic et al., 2010
) (Lowery and O'Reilly, 2011). This is a key downstream regulator of the transforming growth factor beta (TGF-β) signalling cascade (Massague J et al., 2000), hence loss of SMAD4 renders cells resistant to the growth inhibitory effects of TGF-β (Singh et al., 2011). Mutation of *SMAD4* also occurs in colorectal cancer, and small bowel tumours. The SMAD family of proteins signals through serine threonine kinase receptor complexes that propagate signalling in response to TGF-β (Massague J et al., 2000) (Singh et al., 2011).

The *SMAD4* gene maps to chromosome 18q21 and is lost in over 50% of pancreatic carcinomas (Hahn et al., 1996). The locus coding for *SMAD4* undergoes loss of herterozygosity in >90% of pancreatic cancers and in half the cases biallelic inactivation by homozygous deletion or by missense or nonsense mutations occurs (Hahn et al., 1996). Loss of *SMAD4*, as implicated in earlier studies has been shown to confer a worse prognosis (Tascilar et al., 2001). Tasilar *et al.* analysed 249 resected PDAC specimens and showed that patients retaining SMAD4 expression had a significant survival benefit of about 5 months (Tascilar et al., 2001). This is contrary to Biankin *et al.*, who have published an increase in postoperative survival in for patients whose tumours lacked expression of SMAD4 (Biankin et al., 2002). More recently, the association between SMAD4 expression in tumours and patterns of disease spread has been examined (Crane et al., 2011) (Iacobuzio-Donahue et al., 2009). Metastasis was seen in 71% of patients

who lost SMAD4 expression compared to 27% in patients who retained expression of this gene (Crane et al., 2011). Moreover, these findings concur with autopsy data from patients with advanced pancreatic cancer which showed that in metastatic cases 75% of patients had a loss of *SMAD4* compared to 22% of patients who had no metastasis but only localised spread (Iacobuzio-Donahue et al., 2009). This data suggest that *SMAD4* loss predicts for local pattern of disease progression and therefore may allow for selection who would benefit from local therapies (Iacobuzio-Donahue et al., 2009). *SMAD4* has been designated a 'progression allele' as it is necessary for the maintenance of PDAC occurring later in PanIN lesions (Wilentz et al., 2000). Loss of *SMAD4* is present in metastastic cancer indicating the possible role in this process both directly and indirectly (Hruban and Adsay, 2009).

SMAD family of proteins

The SMAD family compromises three subcategories: receptor regulated SMADs (R-SMAD), common partner SMADs (Co-SMADs) and inhibitory SMADs (i-SMADs) (Massague et al., 2000; Truty and Urrutia, 2007b). The R-SMADs are SMAD2 and SMAD3, which are directly phosphorylated by TGF-β receptors upon activation by TGF-β cytokine. Upon activiation, the phosphorylated R-SMADs then associate forming hetero-oligomeric complexes with Co-SMADs. There is only one member of the Co-SMAD group which is SMAD4. Complex of R-SMADs (SMAD2 and SMAD3) and Co-SMAD (SMAD4) are then able to translocate to the nucleus where they enable regulation of both positive and negative transcriptional complex formation for differentiation,

growth suppression and apoptosis (Kitamura et al., 2007; Singh et al., 2011). Inhibitory SMAD (SMAD-7) is normally present in the nucleus at basal levels and is thought to inhibit signalling by inhibiting phosphorylation of TGF-β receptors by binding to them (Schmierer and Hill, 2007). This is thought to be activated through a negative feedback loop with continual stimulation of TGF-β leading to transcription of SMAD7 thereby ensuring a negative feedback.

SMAD4 as an independent functioning molecule

SMAD4 is characterised as a transmitter of signal for the TGF-β superfamily of cytokines, whose widely expressed member TGF-β1, is a potent growth inhibitor. It was therefore assumed on discovery of SMAD4 that loss of TGF-β growth inhibitory response was a result of mutation in SMAD4 expression (Stuhler et al., 2006)which has been challenged with multiple reports in the literature to suggest that these are independent processes (Lowery and O'Reilly, 2011; Singh et al., 2011; Volmer et al., 2004). Evidence from various groups suggest that SMAD4 has a wider independent function in carcinogenesis supporting a broad array of transcription factors controlling important tumour biological processes, for example apoptosis, epithelial mesenchymal transition and cell migration (Kitamura et al., 2007) (Schwarte-Waldhoff et al., 1999) (Zapatka et al., 2007). These not only have a bearing on the cancer cell protein expression but also interconnect SMAD4 signalling with other signalling mechanisms in the crosstalk process with the surronding stroma (Kitamura et al., 2007; Sheikh et al., 2007).

In experiments utilizing human pancreatic cell lines, restoration of SMAD4 in negative cells lines, demonstrates that SMAD4 can control the angiogenic switch (Schwarte-Waldhoff et al., 2000). *SMAD4* restoration in cells produced an inhibition of angiogenesis by decreasing expression of VEGF and increasing expression of thrombospondin-1, as well as reducing invasion and possibly ECM remodelling through downregulation of MMP-2 and MMP-9 (Schwarte-Waldhoff et al., 2000). In colonic cancer cell lines SW480 re-expression of *SMAD4* resulted in morphological reversion in the cell from mesenchymal like spindle shaped cells to an epithelial phenotype (Muller et al., 2002). In addition to this, the invasion suppressor gene *E-cadherin* was also induced in these cells, indicating that *SMAD4* has tumour suppressor functions. A more recent study has indicated that SMAD4 also targets genes, involved in the extracellular matrix generation. Breachment of the basement membrane is usually brought about by epithelial and stromal cells and is a hallmark of invasive carcinomas (Zapatka et al., 2007). Laminin-5 is a one such heterodimeric epithelial-derived basement membrane component, commonly lost in carcinomas but not in insitu tumours. In human colon and pancreatic tumour cells, SMAD4 functions as a positive transcriptional regulator of all three genes encoding laminin-5. This transcriptional activity was lost in SMAD4 negative cell lines and re-expression of the three laminin-5 chains was induced by reconstitution of SMAD4 (Zapatka et al., 2007).

Proteomic analysis of conditioned media and cell lysate from SMAD4 re-expressing colorectal and pancreatic cancer cell lines has shown differentially expressed proteins in both groups compared to their respective SMAD4 negative counterpart. Furthermore, up-

and downregulation of certain proteins has also been demonstrated (Volmer et al., 2004) (Volmer et al., 2005) (Stuhler et al., 2006). Analysing the secretomes of SW-480 colonic cancer cell lines demonstrated that expression of SMAD4 in the cancer cell lines was suppressed by the expression SPRAC which is a secreted matricellular macromolecule exhibiting a host of biological functions ranging from cell adhesion, tissue remodelling, proliferation and motility (Volmer et al., 2004). Proteomic analysis of SW-480 colonic cancer cell lines using two dimensional gel electrophoresis, has determined over 47 proteins, which have a SMAD4 dependent expression (Stuhler et al., 2006).

Cytokine profiling of pancreatic cancer cells and specimens has been undertaken by Bellone *et al.* analysing their prognostic relevance, however differential cytokine expression based on their SMAD4 status was not demonstrated (Bellone et al., 2006). Kitamura and colleagues have also used a mouse model to investigate the role SMAD4 has on influencing the surrounding cellular microenvironment. Experiments with mutant mouse models in which the *SMAD4* gene has been knocked out, accumulated CD34 positive myeloid cells which express matrix metalloproteinases MMP9 and MMP2 promoting the invasive front (Kitamura et al., 2007). *SMAD4* forms a key regulator of tumour progression and cellular cross talk; its role however is not fully understood and is a matter of investigation. Additionally, the influence other mediators have on affecting *SMAD4* mutation and loss in tumours cell is also a subject of great interest.

3.2 TGF-β

TGF-β is an important cytokine that is involved in a wide range of biological processes. It was discovered in 1983 and named after its ability to 'transform' rat fibroblasts (Anzano et al., 1983). It is primarily known for its capacity as a potent inhibitor of cellular proliferation in normal cells (Bierie and Moses, 2006; Singh et al., 2011). In addition, the TGF-β signalling pathway is responsible for numerous other cellular responsibilities including differentiation, apoptosis, angiogenesis, immunosuppression, and wound healing. The pathogenesis and progression of numerous cancers such as pancreas, colon, breast, melanoma, prostate, gastric, neuroendocrine, gynaecologic, skin, and nervous system malignancies, are reliant on the disruption of normal TGF-β signalling (Truty and Urrutia, 2007a) (Massague et al., 2000). This is essentially a result of the loss of the normal growth inhibitory response of TGF-β.

In humans, there are three expressed ligand isoforms (TGF-β1, TGF-β2, and TGF-β3) encoded by different genes, expressed in a tissue specific manner. They all function through the same receptor signalling systems (Massague, 1998). These isoforms are expressed in epithelial, endothelial, hematopoietic, and mesenchymal cells with TGF-β1 being the most widely expressed. Pancreatic tissue normally expresses TGF-β in both acinar and ductal cells (Yamanaka et al., 1993) and it is found as a secreted molecule in the extracellular matrix (Massague, 1998). TGF-β isoforms signal through a family of receptors (Massague, 1998) which are characterised as type-I TGF-β receptor (TGFRI), type II TGF-β receptor (TGFRII), and the type III TGF-receptor (TGFRIII) with the latter having no intrinsic signalling function by itself (Huang and Huang, 2005). The final

target of TGF-β signalling involves interaction with DNA and subsequent transcriptional control of numerous genes with SMAD mediators transmitting these membranes bound signals to the nucleus.

TGF-β in pancreatic cancer

Important events associated with pancreatic malignant transformation are the activation of oncogenes, inactivation of tumour suppressors and loss of sensitivity to the inhibitory effects of TGF-β. In pancreatic cancer the cancer cells have lost their ability to respond to the growth suppressive effects of TGF-β (Ozawa et al., 2001). Immunohistochemical analysis of pancreatic cancer tissue shows the overexpression of all three isoforms of TGF-β (Friess et al., 1993) with overexpression being associated with advanced stage and poor survival (Friess et al., 1993). Serum levels of TGF-isoforms are elevated in patients with pancreatic cancer compared to matched controls and low circulating levels of TGF have been associated with prolonged survival (Bellone et al., 2006).

Mutations of the TGF-β transduction pathway have been characterised in pancreatic cancer accounting for loss of its tumour suppressive effects. The mutation or deletion of SMAD4 a downstream mediator of this pathway is the most common mutation (Hahn SA and et al, 1996). Inactivating mutations in the *SMAD4* gene and upregulation of the inhibitory *SMAD6* and *SMAD7* genes are therefore thought to contribute towards unresponsiveness to TGF-β signalling (Singh et al., 2011). Furthermore, studies have shown that loss of SMAD4 signalling in T-lymphocytes leads to spontaneous epithelial

tumours throughout the gastrointestinal tract including pancreatic tumours, whereas epithelial specific deletions did not thus suggesting that SMAD4 signalling plays a critical role in the immunologic suppression of gastrointestinal cancer (Kim et al., 2006).

The reports regarding the TGF-β receptors are slightly more conflicting with over-expression being reported in some studies (Friess et al., 1993; Lu et al., 1997) in pancreatic cancer cell lines while other studies reporting under expression (Nicholas FJ and Hills CS, 2003). The genes encoding the human TGF receptors have also been found to be inactivated by mutations. Mutations in the TGFRII gene are found in pancreatic as well as in colon, gastric, liver, and breast tumours (Massague et al., 2000). Most of these are a result of frameshift and result in truncated proteins that lack both the transmembrane and intracellular domains (Markowitz SD and Roberts AB, 1996). Epigenetic regulation of expression control of TGFRII is another way receptor function is lost. Epigenetic mechanisms are changes in gene expression without associated DNA alterations. This is done primarily through defects in transcriptional regulation at the promoter and histone level leading to repression and gene silencing (Truty and Urrutia, 2007a).

The role of TGF β in tumour suppression

The most critical role of TGF-β is its role as a tumour suppressor agent. The inhibition of cellular proliferation is central to TGF-β responses in epithelial, endothelial, hematopoietic, neural and certain types of mesenchymal cells (Massague et al., 2000). TGF-β primarily induces inhibition of cell cycle progression during G1 phase [3]. This is

brought about by inducing the expression of the cyclin kinase inhibitors such as p15, p21, and p27 (Truty and Urrutia, 2007a). These inhibitors block cyclins and CDKs complexes from phosphorylating Rb and thereby inhibiting G1/S progression. In addition, TGF-β also directly suppresses mitogenic c-myc expression (Pietenpol et al., 1990), which is a promoter of cell growth and proliferation. Pancreatic cancer cells and other cancer cells are also susceptible to apoptosis which is induced by TGF-β (Truty and Urrutia, 2007a).

The role of TGF-β in tumour progression

One of the unique aspects of TGF-β lies within its apparent dichotomy of functions. In normal cells, TGF-β1 functions as a potent tumour suppressor however conversely in malignant cells it behaves as a tumour promoter being indicative of a more aggressive phenotype (Glynne-Jones et al., 1994). In addition, high levels of this cytokine can be produced in the tumour stroma and microenvironment (Sieweke and Bissell, 1994). The tumour promoter activity is due to the overexpression of TGF-β1 itself directly affecting the cancer cells, however it is also attributed to several other causes. These are altered expression of ECM components, the stimulation of angiogenesis, and the immunosuppressive effects (Massague et al., 2000). Current evidence suggests that this cancer mediated immune evasion is due to the potent immunosuppressive effects of overexpressed TGF-β1 (Wojtowicz-Praga, 2003). Tumour associated TGF-β1 down regulates the host immune system and contributes to the generation of T-suppressor cells with impaired cytotoxic function and inability to mount an anti-tumour response in vivo(Bellone et al., 1999). These cells then act to suppress other lymphocyte populations

leading to an overall dampened response against tumours. TGF-β1 can also inactivate natural killer (NK) and lymphokine-activated killer (LAK) cells. It is also a potent regulator of the ECM and induces the expression of many of its components such as fibrosis (Truty and Urrutia, 2007a). Overexpression of TGF-β1 induces epithelial to mesenchymal transition (EMT) of normal and transformed epithelial cells thus enhancing their migratory ability. This is demonstrated in myofibroblasts and stellate cells in pancreatic cancer (Bachem et al., 2005). Cells losing epxression of SMAD4 show enhanced TGF- β mediated epithelial –mesenchymal tranisition (EMT). This has been explained by the hypothesis that loss of SMAD4 lead to activation of STAT-3 (signal transducter and activator of transcription 3) which contributes towards switiching TGF-β from a tumour supressor to a tumour promoter (Matthaios et al., 2011) (Gaspar et al., 2007). TGF-β1 overexpression by tumour cells causes an increase in the production of other mitogenic growth signals, including PDGF, and FGF in addition to activating SMAD independent proliferative pathways, such as the RAS/MAPK (Elliott and Blobe, 2005) making it a key crosstalk molecule in the tumour microenvironment.

CHAPTER FOUR

MONOCYTES AND MACROPHAGES

4.1 Monocytes and macrophages

Monocytes and macrophages are haematopoietic cells, which are derived from CD34 positive progenitor cells being produced in the bone marrow (Kurahara et al., 2009; Murdoch et al., 2008). Monocytes mature from precursor myelomonocytic stem cells forming pro-monocytes and then monocytes in the bone marrow. This process is brought about by Interleukin (IL-4) and granulocyte and macrophage colony stimulating factor (GM-CSF) (Murdoch et al., 2008; Valenti et al., 2006). Monocytes and macrophages form an essential part of the innate immunity defending the body against microbes. They are recruited to sites of infection and are present as resident cells in some tissue e.g. Kuffer cells in the liver. In addition to this, these cells are also known to participate in the inflammatory process producing a host of cytokines, which possess both pro- and anti-inflammatory roles (Ruffell et al., 2012).

In cancer these cells have a major role in several aspects of tumour biology, including invasion, recruitment of leukocytes, activation of T-cells, angiogenesis, growth and metastasis hence making them an essential regulator in the tumour microenvironment (Coffelt et al., 2009; Joyce and Pollard, 2009; Lamagna et al., 2006; Ruffell et al., 2012; Schmid and Varner, 2007) (Dirkx et al., 2006). Monocytes are continually shed into the circulation from the bone marrow (Lewis and Pollard, 2006) from where they extravasate into tissue and undergo a phenotypic transformation to form "Tissue associated macrophages" (TAMs) (Kurahara et al., 2012a; Lewis and Pollard, 2006; Ruffell et al., 2012). Macrophages recruited to tissues, are distinct from the classical monocytic population which are involved in inflammatory processes hence have been called

"resident monocytes" by some authors to differentiate them from TAMs (Gordon and Taylor, 2005). Monocytes/ macrophages in some cases are seen to constitute a large proportion of the tumour mass up to 80% in some cancers(Ruffell et al., 2012) (Pollard, 2004). They also carry a prognostic value as a high infiltrate of tumour associated macrophages correlate with poor prognosis in over 80% of studies published in a variety of tumour including pancreatic cancer (Bingle et al., 2002; Kurahara et al., 2012b). In colorectal cancers, macrophages have been linked with an improved prognostic benefit with macrophages found at the invasive tumour front (Forssell et al., 2007). In human breast cancers, there is a positive correlation between poor prognosis and the density of tumour-associated macrophages (Lin et al., 2002; Ruffell et al., 2012). More recently, a bone marrow derived subset of monocytes, which are recruited to tumour sites has been described, known as Tie2 expressing monocytes (TEM) (De Palma et al., 2005). Tie2 is Tyrosine kinase with immunoglobulin-like and EGF-like domains. It is an angiopoietin receptor resulting in cell signalling and angiogenesis promotion.

TAMs and TEMs

TEMs are a subpopulation of tumour infiltrating monocytes originally described in mice, with an affinity towards highly vascularised stromal regions. TEMs can make up to 30% of macrophages in the tumour environment in highly vascular tumours and are virtually absent in necrotic tumours (De Palma et al., 2003). The origins of TEMs are unclear with various school of thought in place. Some believe they are derived from specialised monocyte precursors distinct from those which generate TAMs (De Palma and Naldini, 2009 1765), whereas others believe there are microenvironmental factors in play which

can induce the TEM phenotype in extravasated monocytes or even in TAMs. Studies have shown that TEMs in humans express CD14, CD16 and CD11c surface markers that are similar to the so-called "resident monocytes" indicating the link between TAMs and TEM cells (Gordon and Taylor, 2005). It is clear that TEMs are involved in the angiogenesis process however their exact role is still under review.

Macrophages and in particular TAMs are highly versatile and multifunctional components of the innate immune system and have therefore gained significant importance in the carcinogenesis process of a variety of cancers (Ruffell et al., 2012; Solinas et al., 2009). Macrophages exhibit a variety of phenotypes and functions depending on the physiological and pathological conditions they are recruited to, with ability to secret growth factors, cytokines, proteases and complement components (Lamagna et al., 2006). Macrophages are characterised into 2 broad categories as type I (M1) and Type II (M2), on the basis of their activation states, receptor expression and cytokine production (Joyce and Pollard, 2009; Kurahara et al., 2009; Ruffell et al., 2012; Sica et al., 2006). Type I macrophages are cells which are capable of producing large quantities of proinflammatory cytokines, expressing high levels of MHC molecules and implicated in killing pathogens and tumour cells. Type II (M2) macrophages exhibit a moderate inflammatory response with their function mainly focused at eliminating cell waste, promoting angiogenesis and tissue remodelling (Sica et al., 2006). TAMs belong primarily to the M2 population of macrophages (Kurahara et al., 2009) (Lamagna et al., 2006). The macrophage subpopulation (M1 and M2) have different types of receptor expression and cytokine profile. M1 macrophages express high levels of IL-12 and IL-23

and low levels of IL-10, in contrast to M2 macrophages whose expression of interleukins is entirely opposite the former (Ruffell et al., 2012; Sica et al., 2006). Tissue macrophages express a variety of surface markers with CD68 being the most well known to be expressed in TAMs. As TAMs exhibit the M2 phenotype, they also express CD163 and CD204 (Kurahara et al., 2012b; Pollard, 2004). In pancreatic cancer Kurahara *et al.* have shown that the M2 type TAMs, which express CD163 and CD204, conferred a worse prognosis exhibiting increase lymphatic metastasis. This was not demonstrated when correlations of metastasis and survival were made with CD68 expression in pancreatic cancer (Kurahara et al., 2009). The same author has very recently demonstrated the presence of folate receptor expressing TAMs in pancreatic cancer, which confer a high incidence of haematogenous metastasis and poor survival (Kurahara et al., 2012b). This indicates that heterogeneity is seen in infiltrating tissue macrophages which has been suggested in the recent literature (Ruffell et al., 2012).

A variety of subpopulation of TAMs exist in a tumour, being placid, they have the ability to adapt and undergo transition based on the location and requirements from these cells (Joyce and Pollard, 2009 1674). The ability of macrophages to assume varying roles and phenotypes seems to be influenced by tumours through the process of cellular crosstalk. When monocytes are exposed to anti-inflammatory molecules IL-4, IL-10, glucocorticoid and TGF-β they develop into M2 type macrophages (Siveen and Kuttan, 2009; Solinas et al., 2009).

Macrophages normally provide support for developing tissues through their matrix remodelling capacities, synthesis of growth and angiogenesis factors. They have the ability to engulf debris and apoptosis. A detailed role of monocytes and macrophages in cancer is described in the sections to follow.

Recruitment of monocytes / macrophages to tumour

Chemotactic products produced by stromal and tumour cells drive monocytic and macrophage recruitment. These primarily include the chemokines (CC), CCL2 also known as MCP-1 (monocyte chemotactic protein-1) and CCL5 (RANTES) which are key regulators (Coussens and Werb, 2002). In addition increased levels of CCL3 (macrophage inflammatory protein-alpha MIP-1A), CCL4 (MIP-1B), CCL8 (MCP-2), placental growth factor (PIGF) , TNF-α, TGF-β, colony stimulating factor (CSF), vascular derived growth factor (VEFG) are also found in the tumour microenvironment serving as chemoattractants (Coffelt et al., 2009; Fischer et al., 2007; Scotton et al., 2001; Siveen and Kuttan, 2009; Wu et al., 2008). Moreover, monocytes could be attracted by fibronectin, fibrinogen and other factors produced during the cleavage of ECM proteins induced by macrophage and/or tumour cell-derived proteases (Coffelt et al., 2009). Monocytes once recruited to the tumour environment can be grouped into different subsets based on their receptor expression and can be either inflammatory or resident monocytes, which will go on to form M1 macrophages and M2 macrophages respectively. This is an important process, as the type of macrophage developed will have a bearing on the tumours growth and invasive potential. It has been shown that low levels

of CCL2 production by the tumour corresponds to a modest infiltration of monocytes leading to tumour growth due to selection of M2 TAMs. On the other hand, high levels correspond to increased M1 TAM development leading to destruction of the tumour. This would suggest the biological effect of CCL2 is biphasic depending on the levels of secretion of the chemokine (Nesbit et al., 2001). Interestingly TEMs do not express receptors for CCL2 and therefore do not respond to this chemokine suggesting their recruitment is guided by different chemokines. Monocytic recruitment is achieved thorough chemokines CCL3, CCL5 and CCL8 ,through chemokines receptors CCR1 and CCR5 (Murdoch et al., 2008) present on the monocytes. More recent studies indicate that TEMs are recruited by the Tie2 ligand angiopoietin-2 which is produced by hypoxic tumours and endothelial cells (Gu et al., 2006).

CCL5 is produced by T-cells, however it is also produced by certain tumour cells such as breast carcinoma cells, contributing to monocytic migration and also TEMs recruitment. It also induces the monocytes to express CCL2, CCL3 and CCL4 allowing for an autocrine effect (Locati et al., 2002). Similarly CSF-1 is produced by macrophages and monocytes and is involved in their recruitment being a key regulator of this function as experiments with transgenic mouse models show that the CSF-1 null mutant have a marked reduction in monocytic infiltration(Lin et al., 2001). VEGF expressed in macrophages serves as a chemoattractant for monocytes via activation of VEGF receptors VEGF-R1, as evidenced by data showing that neutralizing antibodies against VEGF reduce migration of monocytes (Bingle et al., 2002). VEGF is mainly expressed in areas

of hypoxia and as TAMs express high levels of VEGF they tend to accumulate in necrotic and poorly vascularised tissue (Ruffell et al., 2012).

A group of molecules known as alarmins has also been under the spotlight regarding their role in monocytes recruitment (Ehrchen et al., 2009). High mobility group box protein-1 is one such protein which is seen to attract monocytes and macrophages alike, being released in areas of necrosis and by dying tumour cells (Schlueter et al., 2005).

4.2 Role of macrophages and monocytes in the tumour microenvironment

Role in Tumour proliferation and metastasis

It is now clear that stromal cells are drivers of variety of functions, which aid in tumour growth, spread and invasion. Tumour infiltrating myeloid cells (macrophages and monocytes) have a multi-faceted role in this process. Primarily myeloid cells have important and well-studied roles in tumour proliferation, metastasis and angiogenesis.

Mouse model experimentation has confirmed their prometastatic role, as neutralizing antibodies to chemoattractants leads to a lack of monocytic / macrophages recruitment into tumours, resulting in impaired metastasis (Lin et al., 2001). In vivo mouse model experiments, it has been observed that monocytes when coengrafted with human tumour cells, tumour growth enhanced, with lesser growth seen when a low ratio of human monocytes was co-grafted. Also repeated contact of monocytes with tumour cells leads to decreased production of cytotoxic molecules (TNF-α, reactive oxygen intermediates, and

IL-12) and increased production of immunosuppressive cytokine (IL-10) (Baj-Krzyworzeka et al., 2004). Experimental and histological evidence has indicated the presence of monocytes in areas of basement-membrane breaches and also at the invasive front in more advanced tumours (Lin and Pollard, 2004).

Experiments utilizing mammary tumour cells by Condeelis *et al.* indicate that TAMs and tumour cells closely interact, with TAMs producing epidermal growth factor (EGF) and tumour cells producing CSF-1 to simulate migration of each other (Condeelis and Pollard, 2006; lyWyckoff et al., 2007). This reciprocal crosstalk can be blocked resulting in decreased migration and invasion of both cell types (Wyckoff et al., 2004). The group has also shown, using multiphoton imaging, that there is a presence of macrophages at the tumours invasive front. It has also been determined that macrophages aid in the invasion of epithelial cells at the tumour front (Pollard, 2004). Macrophage derived tumour necrosis factor TNF-α increases the invasiveness and motility of breast and ovarian cell lines inducing the production of matrix metalloproteinases in them (Schoppmann et al., 2002; Solinas et al., 2009). This has a paracrine effect on macrophages, stimulating them to produce MMP-2 and MMP-9 that aids in metastasis (Solinas et al., 2009). In pancreatic cancer cell lines motility is increased by the presence of TNF-α acting either directly or through the paracrine mechanisms that is described above (Baran et al., 2009). MMP-7 is also upregulated in macrophages in areas of tumour hypoxia, which controls downstream regulation of prometastatic factors aiding tumour progression (Coffelt et al., 2009; Lynch et al., 2005). Hiratsuka *et al.* have illustrated that

macrophages produce MMP-9 in response to tumour VEGF at sites of distant metastasis, hence aiding tumour spread (Hiratsuka et al., 2006).

Macrophages are a major producer of proteases, allowing tumour cells to invade bloods vessels and breach the basement membrane. These include specific metalloproteinases, cathepsins and serine proteases (Egeblad and Werb, 2002; Mohamed and Sloane, 2006). These proteases enable tumour cell mobility by cleaving cell surfaces proteins such as E-cadherin, degradation of ECM proteins and activation of growth factors and cytokines through cleavage of active domains. Monocytes secrete MMP-19 and urokinase plasminogen activator (uPA) which allows tumour cells to detach and disseminate leading to distant metastasis (Robinson et al., 2002). Protease-enhanced invasion is not an individual phenomenon but is regulated through a cascade of interactions working together. Being potent effectors of tumour invasion and growth, macrophages hold a central position and are the subject of future therapeutic modalities.

Role in Angiogenesis

Promotion of angiogenesis leading to cancer growth and metastases is a well established role of monocytes and macrophages having been studied in a variety of tumours. Clinical evidence show a correlation between local macrophage density and areas of intense angiogenesis defined by the presence of microvessels, leading to poor prognosis (Murdoch et al., 2008; Solinas et al., 2009; Valkovic et al., 2002). In the mouse breast cancer model, macrophages (TAMs) were expressed in high numbers even in

premalignant lesions and seen to initiate the "angiogenic switch" (Lin et al., 2006). This is identified as the formation of a high-density vessel network and is closely associated with the transition to malignancy. The angiogenic switch was delayed if inhibition of macrophage infiltration occurred. This study shows that TAMs play a key role in promoting tumour angiogenesis, an essential step in tumour progression from insitu to invasive malignancy (Lin et al., 2006).

TAMs release a host of proangiogenic growth factors such as VEGF, TNF-α, PDGF, TGF-β, members of the FGF family, cyclooxygenase-2, plasminogen activator, urokinase and metalloproteinases MMP7, MMP9, and MMP12 (Murdoch et al., 2008; Solinas et al., 2009),(De Palma and Naldini, 2009). Macrophages also produce the angiogenic factor thymidine phosphorylase, which in vitro promotes endothelial cell migration (Solinas et al., 2009). Macrophages tend to accumulate in areas of hypoxia or necrosis in numerous tumours such as breast, colon, ovary, bladder (Murdoch et al., 2008). Hypoxia promotes HIF-1 and HIF-2 transcription factors in macrophages whose targets include genes of proangiogenic factors such as VEGF, endothelins and those of proliferation, and metabolism (Lewis and Murdoch, 2005). Macrophages also produce a potent proangiogenic enzyme MMP9 which is responsible for degradation of ECM and promotion of angiogenic factors; inhibition of the which blocks the release of VEGF and thereby inhibiting angiogenesis (Coussens et al., 2000) (Condeelis and Pollard, 2006). Similarly microarray studies on pancreatic carcinoma sections have shown an increased infiltrate of mast cells and macrophages which were not only indicative of a poor prognosis but resulted in high levels of tumour stromal cellular expression of VEGF-A,

VEGF-C and FGF (Esposito et al., 2004). Direct blockage of VEGF in a process to control tumour growth and angiogenesis has met with varying levels of success with Bevacizumab being successful in treatment of colorectal cancer but has no role in pancreatic cancer (Dineen et al., 2008). Dineen *et al.* have demonstrated that 2C3, a monoclonal antibody to VEGF prevents it from binding to VEGFR-2, has been effective in reducing the number of tumour-associated macrophages in pancreatic cancer treatment resulting in decreased macrophage population and subsequent angiogenesis (Dineen et al., 2008).

It is interesting to note that MCP-1 expression correlates significantly with levels of VEGF, TNF-α, and IL-8, suggesting a possible role for MCP-1 as an angiogenesis regulator as well (Dirkx et al., 2006). The expression of chemokines involved in angiogenic processes is also regulated by chemokines such as CCL2, CXCL8, CXCL1, CXCL13, and CCL5. Levels of CXCL5 and CXCL8 were associated with increased neovascularisation and correlated inversely with survival (Balkwill, 2004; Luster, 1998; Mantovani et al., 2004; Strieter et al., 2004). Thus, TAMs have the capacity to affect each phase of the angiogenic process including degradation of extracellular matrix, endothelial cell proliferation, and endotheital cell migration. Transgenic mouse model experimentation in which Tie2 expressing cells were killed using the drug ganciclovir, showed a profound reduction in angiogenesis and growth in tumours (De Palma et al., 2005). Ablation of TEMs from these tumours did not affect the population of TAMs, which would suggest that rather than giving rise to them TEMs are a distinct subpopulation cells. The roles of how TEMs effect angiogenesis in human tumours is still

under investigation however in murine models TEMs produce bFGF and proteolytic factors such as MMP9, however by and large their exact functioning is still a matter of investigation(De Palma and Naldini, 2009) (De Palma et al., 2005)

Summary of the role of macrophages and monocytes

The Figure 1.5 demonstrates a schematic representation of the role of macrophages and monocytes in tumour biology and the relationship they have with, stromal and tumour cells.

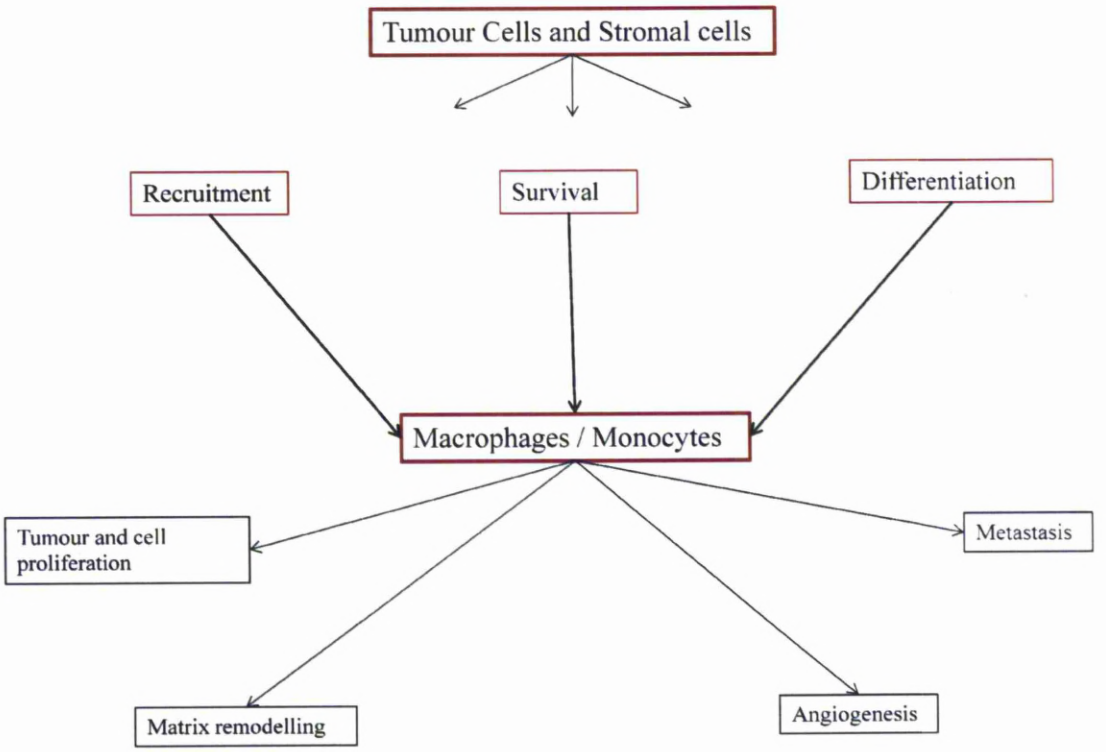


Figure 1.5: Schematic presentation of monocytes and macrophages involvement in cancer progression and cell and complex crosstalk between cells.

CHAPTER FIVE

CALCIUM BINDING PROTEINS

5.1 S100 Family of Calcium binding proteins

Calcium ions form an important intracellular mediator to a variety of secondary messengers, which either can enter the cell through calcium channels in cell membranes or be mobilised from internally sequestered stores. Calcium ions form parts of a variety of cellular functions which include cell-cycle progression, differentiation, muscle contraction, enzyme activation and apoptosis (Kim E. Barrett et al., 2010). Calcium ions often couple with a variety of proteins, affecting their architecture, resulting in activation of their function, in turn affecting their cellular response (Ikura M et al., 2002).

The commonest protein from this family is that with the EF-hand motif. First described by Kretsinger and Nockolds in 1973 (Kretsinger RH and Nockolds CE, 1973), the EF hand motif is a calcium binding motif comprised to two helices (E and F) which are joined by a loop. It is within this loop region that calcium ion binds (Zimmer DB et al., 1995) resulting in the EF-hand proteins to undergoing a conformational change.

The S100 protein family is a multigene calcium binding protein family which constitute the largest subgroup of EF hand proteins. The family consists of over 20 known members in humans each coded by a separate gene (Schafer BW and Heizmann CW, 1996). First described by Moore *et al.* in 1965 these proteins are small acidic proteins between 10-12 KDa and were termed S100 as they dissolved in 100% saturated ammonium sulphate at neutral pH (Moore BW, 1965; Zimmer DB et al., 1995). Many of the S100 proteins demonstrate tissue and cell specific expression patterns. In addition to this they are unusual in that they also occur in the extracellular space, where they are thought to act

like cytokines (Marenholz et al., 2004). In the recent years they have been linked with various human pathologies including inflammatory and neoplastic disorders (Ghavami et al., 2008b{Gebhardt, 2006 #1084; Salama et al., 2008). They also have critical roles in signal transduction pathways including the RAGE pathway (receptor for advanced glycation pathway) (Ghavami et al., ; Ghavami et al., 2008b; Nacken et al., 2003).

In addition to calcium, many of the S100 proteins also display high affinity for zinc and copper ions and this may influence their activity in the extracellular space (Heizmann CW and Cox JA, 1998). Further evidence and indication of their involvements in inflammatory and neoplastic disorders is that most S100 genes are found near the breakpoint region of chromosome 1q21 which when affected results in a number of genetic abnormalities related to autoimmune pathologies or cancer (Mischke et al., 1996).

The S100 proteins have the ability to form homodimers and heterodimers (Barger SW et al., 1992). The S100 proteins (Figure 1.6) have two distinct EF hands, one common to all EF proteins in the C-terminus and one specific to this family on the N-terminus. In addition, beyond the C-terminus in EF hand region is a stretch of amino acids referred to as the C-terminal extension, which is variable in different S100 proteins accounting for varying properties and biological function (Nacken et al., 2003) (Salama et al., 2008).

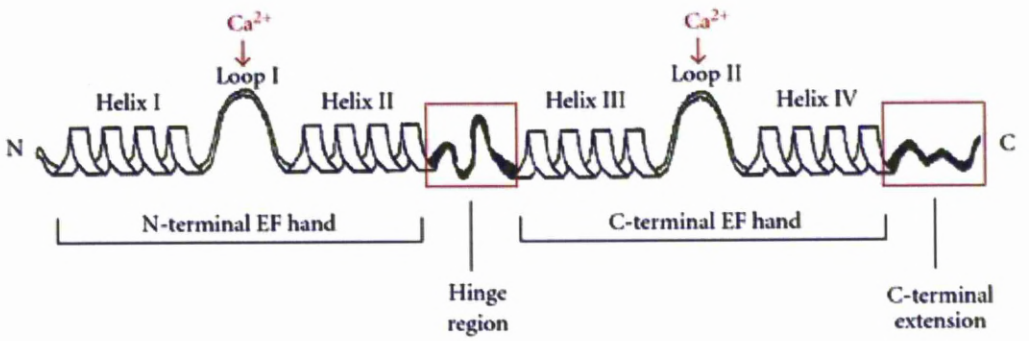


Figure 1.6: Schematic depiction of the secondary structure of an S100 protein. The monomeric structure consists of a repetitive EF-hand motif. The N-terminal and the C-terminal EF hands are connected by a linker region (hinge region). The hinge region and the C-terminal extension (boxed in red) display the least amount of sequence homology among S100 paralogs.

Function of S100 Proteins

The S100 proteins are involved in a broad range of cellular processes, including cell cycle regulation, cell growth, cell differentiation and motility. Intracellular functions include protein phosphorylation, enzyme activation, calcium homeostasis, regulation of cytoskeletal components and regulation of transcriptional factors (Santamaria-Kisiel et al., 2006). A number of S100 proteins interact with p53 with both S100B and S100A4 being the most relevant (Grigorian et al., 2001). Both of these proteins have been shown

to inhibit p53 phosphorylation leading to inhibition of transcriptional activity inducing p53 related cell growth arrest (Scotto C et al., 1998) (Grigorian et al., 2001). In contrast, S100A2 promotes p53 transcriptional activity thus allowing for a balance of actions, which are regulated by different members of the S100 family within a single cell (Mueller et al., 2005). S100A4 has been shown to have a role in cytoskeletal modulation because of interactions with tubulins, actin and myosin filaments producing cell motility and in the cancer setting modulating metastasis (Grigorian et al., 2001). Primarily S100A1, but also to lesser degree S100B, stimulate calcium release in skeletal muscle cells producing cell contractility (Fano G et al., 1989). S100A10 forms a natural ligand of annexin II and binding with the protein induces co-localisation of annexin II (Thiel C et al., 1992) and inhibits its phosphorylation regulating annexin II activity and interaction with cytoskeleton constituents. In pancreatic cancer, Nedaji *et al.* reported co-localisation of annexin II and S100A6 in the plasma membrane and a direct association of levels of membranous annexin II expressions and cytoplasmic S100A6 expression (Nedjadi et al., 2009).

Extracellular effects have been described for S100B, S100A1, S100A2, S100A4, S100A7, S100A10 and S100A12 with secretion noted to occur for S100B, S100A8 and S100A9 (Donato, 2003). The S100A8/A9 complex is secreted by neutrophils and monocytes by a novel secretion pathway relying on an intact microtubule network (Ryckman et al., 2003) acting as a potent chemotactic agent for other macrophages and inflammatory cells (Vogl et al., 2004). In addition, these complexes are also detected at sites of acute and chronic inflammation, where it may have a role in leukocyte trafficking

(Ryckman et al., 2003). S100A10 binds tissue type plasminogen activator (tPA), plasminogen and plasmin, and stimulates the tPA dependant conversion of plasminogen to plasmin, and thus may play an essential role in the inhibition of fibrin clot lysis (Kang HM et al., 1998).

S100 Protein expression in Cancer

Several members of the S100 family are differentially expressed in a variety of human cancers conferring a prognostic benefit. S100B, S100A2, S100A4, S100A6, S100A7 and S10011 (Salama et al., 2008) have been studied in detail with S100A4 having attracted the most attention, particularly in colorectal cancer where it has been associated significantly with survival (Gongoll S et al., 2002). Their role in carcinogenesis is variable and at times not fully understood however, primarily they are involved in regulation of cellular processes of cell cycle progression and differentiation (Donato, 2003). The S100 family gene cluster is at chromosome 1q21 and is frequently disrupted in different tumour types, resulting in differential expression of these proteins, hence providing a link between S100 protein and neoplasia (Schafer BW and Heizmann CW, 1996).

S100B protein is overexpressed in melanoma with high levels being associated with metastasis. Increased serum levels corelate to poor survival reflecting tumour load, stage and prognosis (Harpio and Einarsson, 2004) is being used as a valuable marker to assess

patients response to treatment. To a lesser extent this protein is also detected in thyroid and renal cell carcinomas (Ghavami et al., 2009).

Elevated levels of tumour expression of S100A4 of both protein and mRNA are seen in bladder (Salama et al., 2008), colorectal (Gongoll S et al., 2002), breast (Rudland et al., 2000), thyroid (Helfman et al., 2005), pancreas (Rosty et al., 2002)and prostate cancer (Gupta et al., 2003). The expression of S100A4 has been related to an aggressive tumour phenotype yielding a poor prognosis particularly in colorectal and gastric cancers (Gongoll S et al., 2002). Overexpression of S100A4 in mouse model experiments has shown S100A4 to promote metastasis by affecting metalloproteinase activity and cell motility (Mathisen et al., 2003). S100A4 and S100B are thought to inhibit p53 transcriptional activity by inhibition of its phosphorylation and therefore affecting its tumour suppressor activity (Grigorian *et al.*, 2001). Kim *et al.* have demonstrated S100A4 to have a prognostic benefit in predicting relapse of gastric cancer following curative resection (Kim et al., 2008).

Unlike the rest of the S100 family of proteins which show an up regulation in malignancy S100A2 is down regulated in many cancers including melanoma (Maelandsmo et al., 1997), lung (Smith et al., 2004) oral and breast (Liu et al., 2000) cancers. This however is not always the case as this protein is overexpressed in oesophageal and non small cell lung carcinoma (Salama et al., 2008) and the mechanism of how the protein serves as a tumour suppressor and a tumour promoter is not fully understood. The down regulation of S100A2 protein in lung cancer occur early in lung carcinogenesis and therefore has a

potential place as a biomarker of early change in this process (Feng et al., 2001). Another significant S100 proteins which is expressed in cancer is S100A7. High levels of expression were seen in psoriatic keratinocytes (Emberley ED et al., 2003). It is also overexpressed in ductal breast cancer cell lines as well as invasive ductal breast cancer and ductal carcinoma insitu; its presence bearing a poor prognosis (Emberley ED et al., 2003). The precise mechanism through which this protein affects breast cancer is not known, however, there is evidence linking this protein to BRCA1 gene mutation, which is very important in breast carcinoma tumorigenesis (Kennedy et al., 2005).

S100 proteins in pancreatic cancer

There are a number of S100 proteins which are overexpressed in pancreatic cancer including S100A2, S100A4, S100A6, S100A11 and S100P (Vimalachandran et al., 2005) (Cross et al., 2005; Ohuchida et al., 2005) with S100A6 carrying a poor survival benefit. Crnogorac-Jurcevic *et al.* (Crnogorac-Jurcevic et al., 2003) carried out cDNA array analysis of pancreatic cancer tissue and cancer cell lines publishing a detailed account of several S100 genes which are regulated in pancreatic cancer tumorigenesis. The group found that the genes for S100A2, S100A4, S100A6 and S100P were upregulated in pancreatic cancer, whereas the expression of S100A1, S100A7, S100A8, S100A10, S100A11, S100A12 and S100A13 were low. Of the most importance were the S100A6 and S100P genes, which had the highest differential gene expression.

S100A6 protein is overexpressed not only in ductal pancreatic cancer but also in PanIN lesions (Vimalachandran et al., 2005) and intraductal papillary mucinous neoplasms (Ohuchida et al., 2007b) with high level expression linked to poor outcome (Vimalachandran et al., 2005). Depletion of S100A6 in pancreatic cancer cells has proven to decrease invasive potential by decreasing pancreatic cancer cell motility in experimental conditions (Nedjadi et al., 2009). Additionally Nedjadi *et al.* have also shown that knockdown of S100A6 in pancreatic cancer cells is accompanied with a reduction in cells expressing membranous Annexin A2 which is known to effect cell motility.

S100A6 is also upregulated in a variety of tumours other than pancreatic cancer including gastric (Yang et al., 2007) thyroid (Brown et al., 2006) breast (Cross et al., 2005) and colorectal cancers (Stulik et al., 2000) (Komatsu et al., 2000). In colorectal cancer, there is increased S100A6 expression at the cancer invasive front suggesting the role of this protein in invasion and metastasis (Komatsu et al., 2000). Similarly, in cutaneous melanoma cell line experimentation in nude mice, elevated S100A6 cancer expression correlated with high metastatic ability (Weterman et al., 1992).

S100A4 is frequently overexpressed in ductal pancreatic cancer (Rosty et al., 2002) and malignant intraductal papillary mucinous neoplasm of the pancreas (IPMN) (Jang et al., 2009)however pancreatic intraepithelial neoplasia (PanINs) do not shown any expression of this protein (Rosty et al., 2002). In experimental conditions knocking down S100A4 expression using interference RNA strongly suppressed cell growth, induced G2 arrest

leading to eventual apoptosis and additionally decreased cell migration (Tabata et al., 2009). In pancreatic cancer, S100A2 is overexpressed in ductal adenocarcinomas, compared to IPMNs (Ohuchida et al., 2007a). Amongst the pancreatic cancers, ductal carcinomas with poor differentiation show high level of expression of S100A2. Bainken *et al.* have shown that patients having S100A2-negative tumours had a significant survival benefit from pancreatectomy even in the presence of involved surgical margins or lymph node metastases compared to those who expressed S100A2 (Biankin et al., 2009). Recent microarray analyses revealed that expression of S100A11 is upregulated in pancreatic cancer, which is interesting since it is tumour suppressive gene. Analysis by a Japanese group has shown that S100A11 expression is increased in the early stages of pancreatic carcinogenesis and decreased during subsequent progression to invasive cancer (Ohuchida et al., 2006). Similarly, S100P which is a 10.4kDa secreted protein is also expressed in most PDACs and its upregulation correlates to increasing PanIN grade (Dowen et al., 2005). More recently a S100P binding protein has been demonstrated in pancreatic cancer cell lines with downregulation of this protein leading to decreased PDAC adhesion over expression (Lines et al., 2012).

5.2 S100A8 AND S100A9

S100A8 (MRP8, Calgranulin A) and S100A9 (MRP14, Calgranulin B) are calcium binding proteins which have been implicated in a variety of inflammatory pathologies, being predominantly expressed in cells of myeloid lineage (Ehrchen et al., 2009; Ghavami et al., 2009; Srikrishna, 2012). Both S100A8 and S100A9 proteins have low molecular weight, weighting 10 kDa and 14 kDa respectively (Roth et al., 2001).

Expression of S100A8 and S100A9

These proteins are classically expressed in circulating neutrophils, monocytes and immature macrophages and therefore are implicated in a number of inflammatory diseases (Nacken et al., 2003; Odink et al., 1987) (Goebeler et al., 1994; Nacken et al., 2003). Zwadlo *et al.* demonstrated the presence of both S100 proteins in monocytes which were expressed specifically at early stages of monocyte differentiation and downregulated during maturation to macrophages (Cheng et al., 2008) (Zwadlo et al., 1988). The expression of S100A8 and S100A9 changes as monocytes recruited from the blood stream to sites of inflammation, differentiate to mature macrophages (Zwadlo et al., 1988). They initially express both S100A8 and S100A9 and as they mature S100A8 expression ceases leaving only S100A9 which is also subsequently lost as the cell matures further (Zwadlo et al., 1988)(Figure 1.7). Furthermore, these proteins are not expressed in resting tissue macrophages and lymphocytes (Frosch et al., 2004; Nacken et al., 2003; Roth et al., 2003).

Similarly, S100A8 and S100A9 are expressed in mucosal epithelium and epidermis under inflammatory conditions such as psoriasis and malignant disorders (Zucchini et al., 2001). These proteins have also been detected in a variety of tumours being upregulated in breast, gastric, lung, colorectal (Stulik J et al., 1999) and prostate cancers and downregulated in squamous oesophageal cancers (Cross et al., 2005; Gebhardt et al., 2006). More importantly, these proteins form part of the cancer microenvironment having a role in mediating metastatic spread (Hiratsuka et al., 2006) and regulating

tumour cell growth (Ghavami et al., 2009) which is discussed in further detail in the next section.

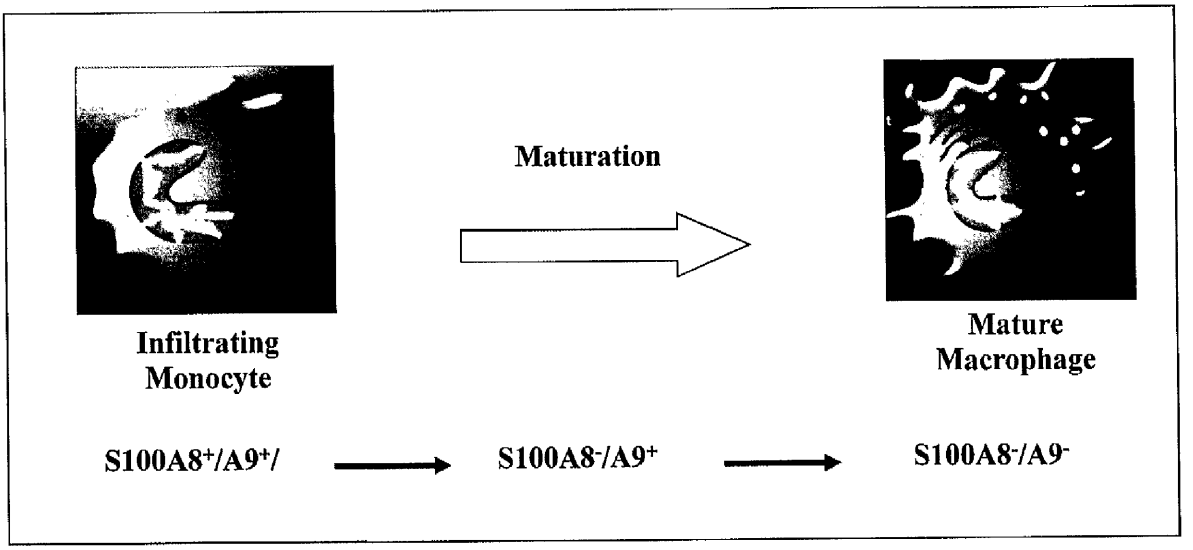


Figure 1.7: Diagram showing the maturation process of monocytes to macrophages and changes in protein expression

S100A8/S100A9 Complex formation and secretion

Although S100A8 and S100A9 exist as homodimers similar to many other S100 proteins, they preferentially form functional anti-parallel heterodimers of S100A8/A9, also known as calprotectin (Ehrchen et al., 2009). In monocytes S100A8 and S100A9 are predominantly found in the cytoplasm being transferred from the cytosol to the

cytoskeleton and then to the plasma membrane on elevation of cytosolic calcium levels (Ehrchen et al., 2009; Roth J et al., 1993). They have a strong tendency to form stable heterodimer complexes on the plasma membrane at sites of inflammation(Nacken et al., 2003) (Striz and Trebichavsky, 2004) and these complexes are thought to contribute to the host inflammatory responses (Nacken et al., 2003; Ryckman et al., 2004; Ryckman et al., 2003). Following their migration to the plasma membrane, they appear as non-covalently associated S100A8/A9 heterodimers on the cell surface. The mechanism by which the heterodimer penetrates the plasma membrane and remains anchored in the plasma membrane is still unclear (Cheng et al., 2008; Roth et al., 2003). Both proteins can be simultaneously induced in monocytes and monocytic cell line HL-60 by several mediators, including LPS, TNF-α, IL1α , TGF-β1, IL-1β , IL-10 or IL-22 (Goyette and Geczy, 2010). Upon stimulation, calprotectin is secreted by a novel secretion pathway dependant on a intact microtubule network (Rammes et al., 1997).

Role of S100A8 and S100A9 in Inflammation

S100A8 and S100A9 are primarily expressed in the leukocytes of the inflammatory tissue and in addition to being present in tissue, elevated levels of these protein are also detected in the plasma (Nacken et al., 2003) and can be useful in monitoring the inflammatory response in such diseases (Roth et al., 2001). In rheumatoid arthritis S100A8/A9 are used as clinical markers of disease remission (Berntzen et al., 1989). Compared to C-reactive protein, S100A8 and S100A9 have been shown to have higher sensitivity and specificity in detecting disease remission and response to therapy. Levels of these proteins are

detected in body fluids and histological specimens alike (Kane et al., 2003). In inflammatory bowel disease (Ulcerative Colitis and Crohn's disease) immunohistochemistry of colonic tissue confirms the presence of elevated infiltrating monocytes and neutrophils expressing S100A8 and S100A9 with faecal calprotectin levels being a useful monitor of disease activity (Tibble et al., 2000).

The presence of calprotectin at sites of inflammation (Nacken et al., 2003) (Striz and Trebichavsky, 2004) contributes to the host inflammatory response by mediating leukocyte trafficking, adhesion and migration (Nacken et al., 2003; Ryckman et al., 2004; Ryckman et al., 2003). These proteins also promote monocytic and phagocyte migration (Manitz et al., 2003; Vogl et al., 2004) and the S100A8/S100A9 heterodimer is believed to recruit further monocytes to sites of inflammation (Eue et al., 2000). S100A9 and S100A8/A9 act as regulatory molecules for transendothelial migration of monocytes by enhancing ICAM-1 ligand binding to monocytes and in turn effecting Mac-1 activity (Eue et al., 2000). Secreted S100A8/A9 also has potent antimicrobial properties and works by depriving the microbes of zinc and manganese ions (Corbin et al., 2008). Due to these proinflammatory properties, S100A8/A9 targeting by antibodies is a novel way to treat these conditions. The proteins, particularly S100A8, are susceptible to oxidation by peroxide, hypochlorite and nitric oxide. This susceptibility, combined with expression in monocytes and immature macrophages produces abundant reactive oxygen species during inflammation and indicates that S100A8 and S100A9 may protect tissues against oxidative damage (Goyette and Geczy, 2010).

Role of S100A8 and S100A9 in Cancer

Expression of S100A8 and S100A9 in cancer cells

Recent clinical and experimental data has shown a strong S100A8 and S100A9 up-regulation in breast, lung, gastric, colorectal, and prostate cancer (Gebhardt et al., 2006) whereas a down-regulation is detected for squamous oesophageal carcinomas (Kong et al., 2004). Furthermore, altered S100A9 expression in carcinomas of glandular cell origin, e.g. breast, lung, and thyroid gland, corresponded to poor tumour differentiation (Gebhardt et al., 2006; Kong et al., 2004). Expression of both S100A8 and S100A9 has been observed by immunohistochemistry in invasive squamous cell carcinoma of the uterine cervix and prostate cancer (Cross et al., 2005; Hermani et al., 2005). In case of the latter (Hermani et al., 2005) S100A9 levels were significantly elevated in serum from prostate cancer patients compared to healthy controls and patients with benign prostatic hyperplasia (Hermani et al., 2006). Moreover, both proteins were detected in the cystic fluid and serum of patients with ovarian carcinomas (Ott et al., 2003). S100A9 expression is induced in hepatocellular carcinoma through activation of the NF-κB signalling pathway (Nemeth et al., 2009). Some studies have attempted to correlate S100A8 and S100A9 expression to the degree of invasive and non-invasive potential of the tumour in experimental conditions but have met with conflicting results.

Role of S100A8 and S100A9 in tumour suppression

The S100A8/A9 protein complex also has a role in apoptosis and has cytotoxic effects against various cancer cell lines in experimental conditions (Ghavami et al., 2009). Ghavami *et al.* has shown this occurs through a mitochondrial pathway involving modulation of the balance of pro and anti-apoptotic family of cell regulators e.g. BCL-2 (Ghavami et al., 2008a). In addition to this, the S100A8/A9 protein complex (Calprotectin) also induces apoptosis by exclusion of zinc from tumour cells (Ghavami et al., 2009). These studies indicate that S100A8 and S100A9 elicit anti-tumour responses, and that the cell death pathway mediated by these proteins therefore might provide targets for developing novel therapeutic tools against cancers. However like many other cellular molecules these proteins have a dual role both in cancer suppression and progression and further understanding of the complex relations these proteins have in the carcinogenesis process still remains to be unravelled.

Role of S100A8 and S100A9 in tumour progression

The role of S100A8/A9 has recently received a lot of attention in the literature for its role in tumour progression. Calprotectin, has been shown to induce proliferation of human breast cell lines (MCF-7 and MDA-MB231) and neuroblastoma cancer cell lines (SHEP and Kelly) via the RAGE (receptor of advanced glycation product) pathway (Ghavami et al., 2008b). Activation of RAGE resulted in downstream activation of the PI3K and RAS related NF-κB pathway, which induced growth factors and cytokines leading to cell proliferation. S100A8/A9, increase migration of benign prostate PNT1A cells in a scratch

wound healing assay (Hermani et al., 2006) and similarly promote migration of Lewis lung carcinoma cells and B16 melanoma cells in Boyden chambers experiments (Hiratsuka et al., 2006). They have also been shown (described in more detail in the results section) to increase motility and proliferation of pancreatic and colorectal cancer cell lines (Ang et al., 2010). In breast cancer, induction of S100A8/A9 converts tumour cells into a migratory phenotype, accompanied by increased expression of matrix metalloproteinases that promote tumour invasion (Moon et al., 2008). Furthermore, siRNA-targeted knockdown of S100A8/A9 expression reduces H-RAS induced invasion.

Binding of S100A8/A9 to tumour cells activates the RAGE mediated pathway and in colon tumour cells, enhances expression of *Cxcl1, Ccl5 and Ccl7*genes. The products of these genes promote leukocyte recruitment, angiogenesis, tumour migration, and formation of premetastatic niches (described below) in distal metastatic organs (Ichikawa et al., 2011).

Studies by Roth's group in Germany using a mouse model have defined the presence of a myeloid-derived suppressor cells (MDSCs) which accumulate in tumours and in sites of inflammation leading to suppression of anti-tumour immune responses (Cheng et al., 2008). The molecular mechanism behind this is unclear however; up-regulation of the myeloid-related protein S100A9 enhances MDSC production in cancer. Moreover, mice that were deficient in S100A9 protein mounted a potent antitumor response and rejected implanted tumours indicating a key role of S100A9 protein in MDSC accumulation and tumour growth. This study demonstrates that tumour-induced upregulation of S100A9

protein is critically important for accumulation of MDSCs and reveals a novel molecular mechanism in cancer.

S100A8 and S100A9 have been associated with a sentinel role in cancer metastasis (Hiratsuka et al., 2006) particularly in the development of the "pre-metastatic niche" (Figure 1.8). Using a mouse model, it has been demonstrated that S100A8 and S100A9 expression was induced in myeloid and endothelial cells in the lungs of mice by secreted soluble factors such as TGF-β, TGF-α and VEGF-A. These soluble factors were derived from the distant primary cancer cells (B16 melanoma or Lewis Lung carcinoma) and form part of the premetastatic phase which enables priming of the distant metastatic microenvironment. This priming of the distant metastatic site allowed for microenvironmental changes, enabling migration and implantation of the tumour cells. Additionally, these proteins also increase motility of circulating cancer cells by activation of pseudopodia which accelerate assembly at metastatic focus (Rafii and Lyden, 2006). Systematic inhibition of S100A8 and S100A9 proteins using blocking antibodies inhibited metastatic disease making these proteins an attractive target for drug therapy. Increased numbers of cells bearing the classical markers of murine MDSC have also been identified in premetastatic lungs and liver in tumour-bearing mice (Yan et al., 2010). S100A9-deficient mice show reduced accumulation of these cells in premetastatic sites further substantiating the role for S100A8/A9 in formation of premetastatic niches and accumulation of MDSC.

Recently Ichikawa *et al.* have, in colon cancer mice models, demonstrated elevated levels of S100A8/A9 in sera of tumour-bearing wild-type mice, prior to any evidence of metastasis. This suggests that these proteins are early makers for metastatic disease having potential to amplify pro-tumour responses (Ichikawa et al., 2011). In addition mice lacking S100A9 showed a significantly reduced incidence of colon tumours, tumour growth and metastasis, reduced chemokine levels, and reduced infiltration of myeloid cells. These findings reveal a novel role for S100A8/A9 in activating specific downstream genes associated with carcinogenesis that promote tumour growth and metastasis identifying them as important players in the molecular crosstalk (Ichikawa et al., 2011),

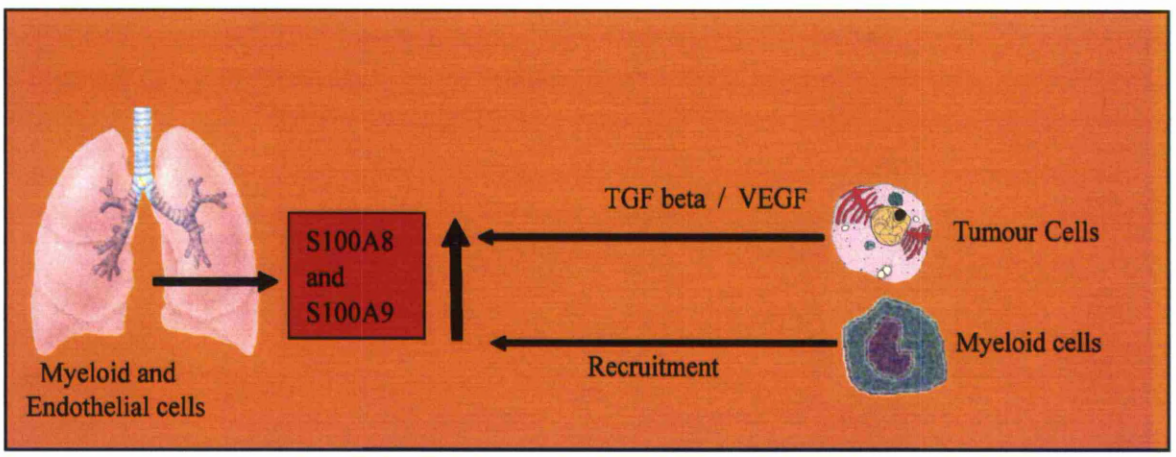


Figure 1.8: Figure adapted from Hiratsuka *et al.* demonstrating the premetastic metastatic phase. TGF-β and VEGF produced by the tumour cells induced the production of S100A8 and S100A9 in lung tissue aiding migration of further myeloid cells to the lung hence preparing the lung tissue for metastatic tumour cell to deposit.

Receptors for S100A8 and S100A9

As eluded in the earlier sections S100A8/A9 signalling is through interactions with cell surface pattern recognition receptors such as Toll-like receptors (TLRs) and RAGE (Srikrishna, 2012). Vogl *et al.* was the first to provide evidence that purified S100A8 bind to TLRs (Vogl et al., 2007). The group also showed that S100A8/A9 amplified endotoxin-mediated inflammatory responses through TLRs. Subsequently, using a mouse autoimmune model, Loser *et al.* (Loser et al., 2010) showed that S100A8/A9 locally present in extracellular medium in tissues of a mouse autoimmune model interacted with TLRs. This interaction increased expression of IL-17, which promoted induction of autoreactive $CD8^+$ T cells, and the development of systemic autoimmunity. In premetastatic lungs of tumour-bearing mice, Hiratsuka *et al.* identified SAA3 as a important downstream effectors of S100A8 and S100A9 ligand for TLR. Therefore, although signalling mediated by a direct interaction of S100A8/A9 with TLR is not implicated, a paracrine cascade consisting of S100A8/ A9, SAA3 and TLR appears of critical importance (Hiratsuka et al., 2006).

RAGE is a transmembrane protein, being a signalling receptor of the immunoglobulin super family. Although originally identified as a receptor for advanced glycation end products, it is known to bind many structurally unrelated ligands. RAGE and S100A8/A9 are co-expressed in tumours and linked to downstream signalling in tumour cells (Ghavami et al., 2009; Ghavami et al., 2008a). Extracellular S100A8/A9 promote the growth of tumour cells, an effect blocked by RAGE gene silencing or by treatment with anti-RAGE (Ghavami et al., 2008b) . Although both TLR4 and RAGE are implicated in

S100A8/A9-mediated pathological effects, which receptor and signalling pathways are preferentially employed may depend on the pathological settings, cell types involved and is still a matter of active investigation.

CHAPTER SIX

BACKGROUND RESULTS

6.1 Results leading towards the project

My projects evolved from findings based on the 2-DE proteomic analysis undertaken by Mr Dale Vimalachandran (MD student under Dr E Costello), comparing similar quantities of microdissected tumour-associated stroma to benign or malignant ductal pancreatic cancer cells (Vimalachandran et al., 2005). He identified two spots with higher intensities in gels containing stromal derived proteins compared to the gels containing proteins from benign or malignant ductal cells. Both of these spots were undetectable in all the benign ductal epithelial cells (n=3) and malignant cells (n=3) cases he examined.

In order to independently confirm the proteomic findings, an immunohistochemical analysis of archival paraffin embedded tissue sections from patients with pancreatic cancer was undertaken. This clearly demonstrated positive immunoexpression in stromal cells rather than malignant ductal cells. These finding were then followed up by staining of a tissue microarray, which held information on 71 patients, which was undertaken by Dr. E Costello.

S100A9 and S100A8 expression in stromal cells

Immunohistochemical staining of a pancreatic cancer tissue microarray for S100A8 and S100A9 resulted in no specific immunostaining of either benign or malignant epithelial cells (Figure 1.9) as mentioned above. In contrast, strong immunostaining was readily detected in the inflammatory cells in the tumour associated stroma (Figure 1.9). Some tumours contained a limited number of S100A8- and S100A9- positive inflammatory

cells while others displayed a high density of positive cells infiltrating the tumour. Positive immunostaining was detected in the stromal component of all tumours (71/71).

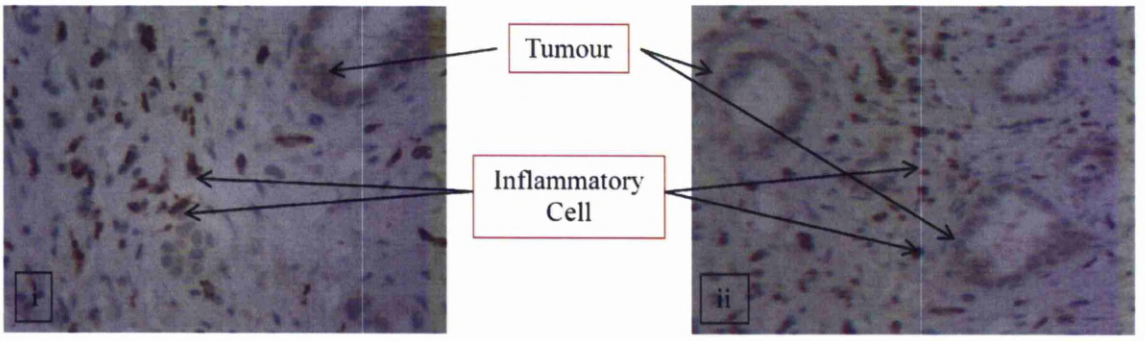


Figure 1.9: Photomicrographs showing sections of malignant tissue, from a pancreatic cancer microarray immunohistochemically stained for S100A8 (left panel) (i) and S100A9 (right panel) (ii) demonstrating their presence in inflammatory cells.

Quantification of S100A8 and S100A9 staining

To evaluate the extent of S100A8 and S100A9 expression in pancreatic tumour-associated stroma Dr E Costello counted the number of positively stained cells in each tumour core of the immunostained cancer microarray. The mean number of positive cells per duplicate patient core is shown in the box plot below (Figure 1.10). Tumours contained significantly fewer numbers of S100A8- positive cells than S100A9-positive cells (Sheikh et al., 2007). The median number of S100A8-positive cells was 15.0 (inter-quartile range 5.2 – 43.7) compared to a median number of 35 (inter-quartile range 20–74.5) S100A9-positive cells ($p<0.0001$, Wilcoxon Signed Rank Test). A strong positive correlation was observed between the number of S100A8- and S100A9- positive cells (Fisher's exact test, $P = 0.0001$)

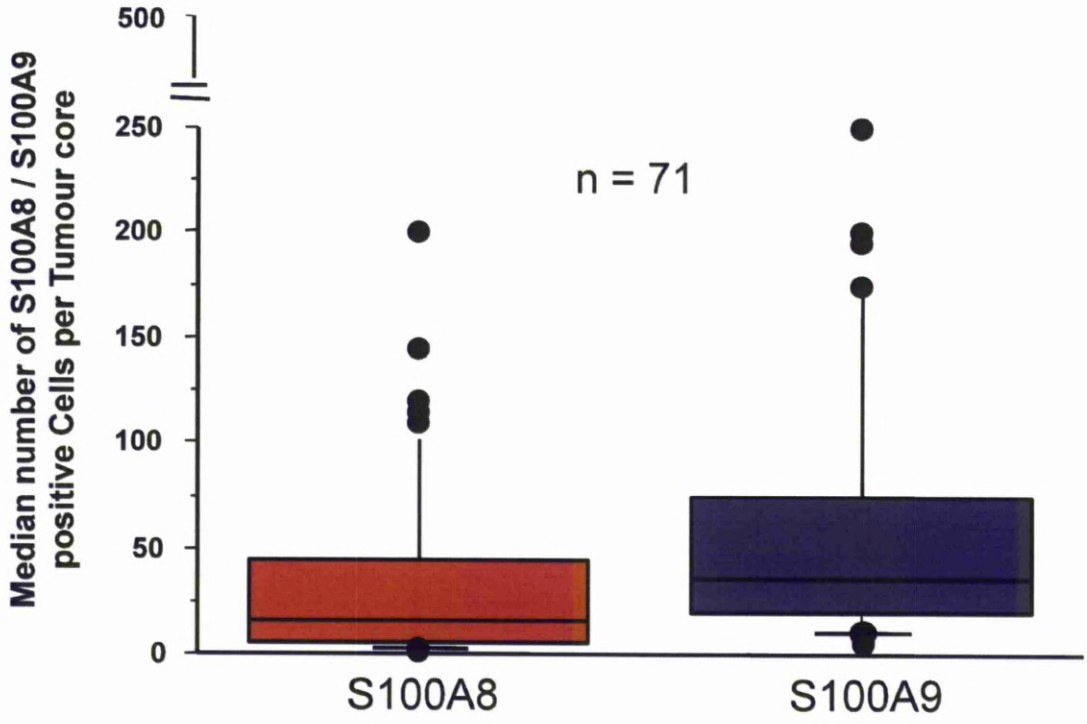


Figure 1.10: Median S100A8 and S100A9 positive cell counts in pancreatic cancer cores on the TMA from 71 patients shown as box plots.

Association of S100A8 and S100A9 expression with patient parameters

Based on the information held in the pancreatic cancer database the number of S100A8 and S100A9 positive cells were correlated with patient parameters. Survival data for all 71 patients was available with no correlation to patient survival (Spearman Rank correlation, *Rho* = -0.08 and -0.02 for S100A8 and S100A9 respectively; P= 0.48 and 0.85 respectively) was demonstrable. For the purposes of examining associations between the number of S100A8 or S100A9 immunopositive cells and other patient

parameters, patients were categorized into two groups, i.e. those having positive cell numbers less or equal to the median or greater than the median for the two respective proteins. Data was available on gender, age at surgery, nodal metastases, tumour size and grade, for all 71 patients. Resection margin status, vascular invasion and perineural invasion data was available for 63, 65 and 68 patients respectively. No associations were observed between the numbers of stromal associated S100A8- or S100A9-positive cells and these clinicopathological parameters (Table 1.3).

Parameters	All Cases n=71 (%)	Low S100A8$^+$ (≤ median) n=36 (%)	High S100A8$^+$ (> median) n=35 (%)	Signif.	Low S100A9$^+$ (≤ median) n=39 (%)	High S100A9$^+$ (>median) N=32 (%)	Signif.
Gender							
Male	43 (61)	25 (69)	18 (51)	ns[1]	23 (59)	20 (63)	ns[1]
Female	28 (39)	11 (31)	17 (49)		16 (41)	12 (37)	
Age at surgery							
<60 yrs	20 (28)	7 (19)	13 (37)	ns[1]	7 (18)	13 (40)	ns[1]
>60 yrs	51 (72)	29 (81)	22 (63)		32 (82)	19 (60)	
Tumour size							
<20mm	22 (31)	13 (36)	9 (26)	ns[1]	13 (33)	9 (28)	ns[1]
>20mm	49 (69)	23 (64)	26 (74)		26 (67)	23(72)	
Tumour grade							
Poorly dif.	25 (35)	13 (36)	12 (34)		15 (38)	10 (31)	
Moderate dif.	36 (51)	15 (42)	21 (60)	ns[1]	17 (44)	19 (59)	ns[2]
Well dif.	10 (14)	8 (22)	2 (6)		7 (18)	3 (10)	
Nodal metastases							
Present	57 (80)	28 (78)	29 (83)	ns[1]	37 (79)	26 (81)	ns[1]
Not present	14 (20)	8 (22)	6 (17)		8 (21)	6 (19)	
Involved resection margin (n=63)							
Yes	42 (59)	22 (61)	20 (57)	ns[1]	24 (67)	18 (56)	ns[1]
No	21 (30)	9 (25)	12 (34)		10 (26)	17 (34)	
Not recorded (n=8)	8 (11)	5 (14)	3 (9)		5 (13)	3 (10)	
Vascular invasion (n=65)							
Present	54 (76)	28 (78)	26 (74)		32 (82)	22 (69)	
Not present	11 (16)	5 (14)	6 (17)	ns[1]	4 (10)	7 (22)	ns[1]
Not recorded (n=6)	6 (8)	3 (8)	3 (9)		3 (8)	3 (9)	
Neural invasion (n=68)							
Present	65 (92)	33 (92)	32 (91)		37 (95)	28 (88)	
Not present	3 (4)	1 (3)	2 (6)	ns[1]	0 (0)	3 (9)	ns[1]
Not recorded (n=3)	3 (4)	2 (5)	1 (3)		2 (5)	1 (3)	

Fishers two sided exact test (significance set at $p<0.05$); ns = not significant

Table 1.3: The association of the mean number of S100A8- and S100A9- expression with clinicopathological parameters, showing no significant correlation.

Association of S100A8 and S100A9 expression with SMAD4 status of the pancreatic cancer cells

SMAD4 expression data was available for 64 patients (array stained and scored by Mr D Vimalachandran) of which 29 patients (45%) were SMAD4 negative and the rest were positive. The number of S100A9-positive cells was independent of the SMAD4 status of tumours (Table 1.4) ($p = 0.21$; Fisher's Exact Test), however the SMAD4 negative tumours significantly correlated with the numbers of S100A8-positive cells ($p = 0.023$; Fisher's Exact Test, Table 1.4).

SMAD4 EXPRESSION	ALL CASES	LOW S100A8$^+$ CELLS	HIGH S100A8$^+$ CELLS	SIGNIFICANCE	LOW S100A9$^+$ CELLS	HIGH S100A9$^+$ CELLS	SIGNIFICANCE
Pancreatic Cancer (n=64)	**n=71**	**n=36**	**n=35**		**n=39**	**n=32**	
Positive	35	19	10	**0.023**[1]	18	19	0.21[1]
Negative	29	12	23		16	11	
Not Recorded	7	5	2		5	2	

[1] Fishers two sided exact test (significance set at $p<0.05$)

Table 1.4: SMAD4 expression and its association with S100A8 and S100A9 expression in the pancreatic cancer stroma.

Interestingly when the median number of S100A8 positive cells per tumour core was examined relative to SMAD4 status, SMAD4-negative tumours had significantly fewer S100A8 positive cells (median 8, IQR 3.0 – 20) compared to SMAD4-positive tumours (median 20.5, IQR 9 - 49; p = 0.01, Mann-Whitney U test). Such a relationship was not established for S100A9-positive cells and SMAD4 status (Figure 1.11). The median number of S100A9-positive cells in SMAD4-negative tumours was 28 (IQR 18.7 – 60), compared to a median of 40 (IQR 23.5 – 98.2, p = 0.21, Mann-Whitney U test) in SMAD4-positive tumours.

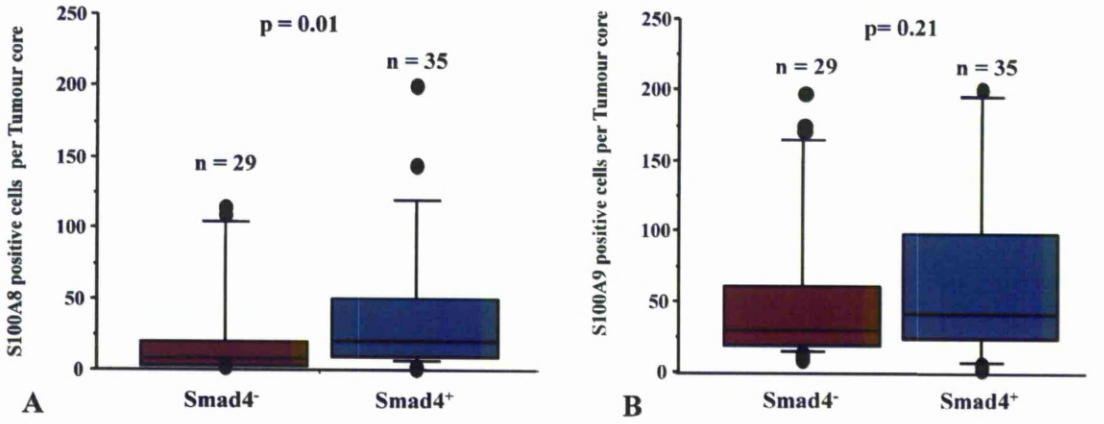


Figure 1.11: The median numbers of S100A8- (A) or S100A9- positive (B) cells per patient were plotted for SMAD4-negative and SMAD4-positive tumours. The p-value shown is for comparison (using the Mann-Whitney U-test) of the median number of S100A8- or S100A9- positive cells for SMAD4 negative and SMAD4 positive tumours.

Moreover, when the ratio of S100A9 to S100A8 cells in SMAD4-positive and SMAD4-negative tumours was examined, a striking difference between the two groups was

noticed. A median of 1.67 fold (IQR 1.21 to 2.4) greater S100A9 to S100A8 positive cells in SMAD4-expressing tumours was observed compared with a median of 3.16 (IQR 1.89 to 6.54) fold greater S100A9 to S100A8 positive cells in SMAD4-negative tumours (p=0.004, Mann-Whitney U test)(Figure 1.12). Thus, a strongly negative relationship between the expression of SMAD4 in tumours cells and the expression of S100A8 in stromal inflammatory cells was established.

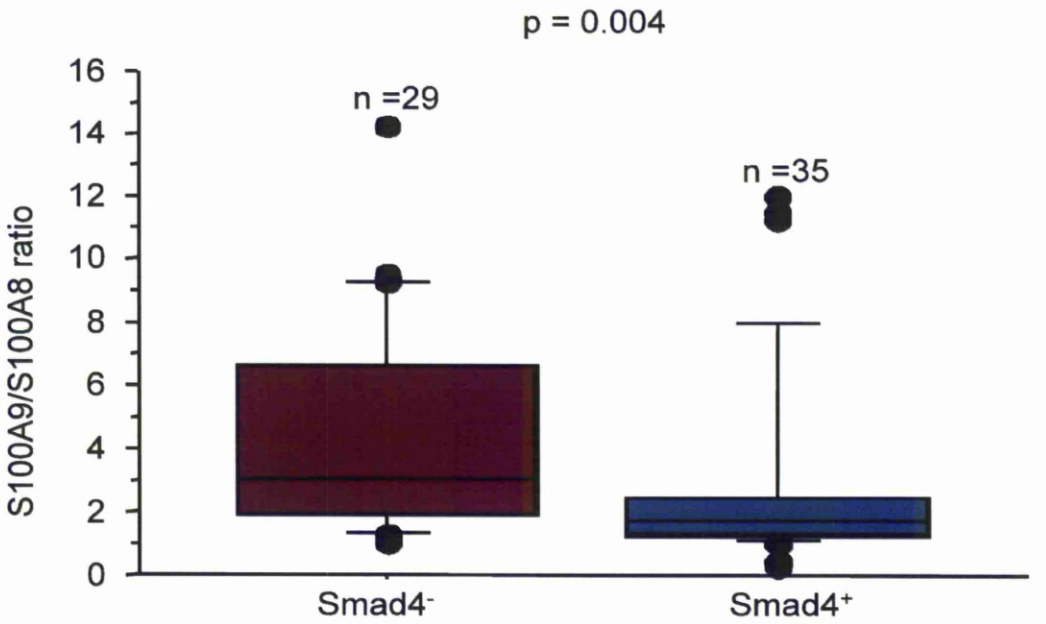


Figure 1.12: Box plot showing the ratio of S100A9 / S100A8 positive cells plotted for SMAD4-negative and SMAD4-positive tumours.

CHAPTER SEVEN

AIMS OF MY RESEARCH

7.1 Aims and objectives of my research

The background results described above demonstrated the presence of S100A8- and S100A9- positive cells in the pancreatic cancer stroma. A relationship between the phenotype of these cells and the tumours SMAD4 status was also uncovered. This indicated the possibility of cellular crosstalk in the pancreatic tumour microenvironment. There has been overwhelming evidence implicating the role of the tumour microenvironment in cancer progression, however there is still an incomplete understanding of the composition and function of the desmoplastic response seen particularly in pancreatic cancer. Furthermore, although it is hypothesised that the cancer progression involves epithelial stromal interactions, the signalling pathways that these interactions employ have yet to be defined. The presence of S100A8 and S100A9 expression in the pancreatic cancer stromal cells and its association with the cancer SMAD4 status allowed for the development of my MD project.

Statement of Aim

To understand the role S100A8 and S100A9 proteins played in the pancreatic cancer microenvironment.

Objectives of my MD project

1. To determine which inflammatory cells in the pancreatic cancer stroma expressed the presence of S100A8 and S100A9 proteins using immunohistochemical and co-immunoflorescence technology.

2. To determine the effects pancreatic cancer cells had on the expression of S100A8 and S100A9 in these stromal cells. This would be undertaken using cancer cell lines and primary human cells in cell culture experiments. Protein expression would be measured using western blotting analysis.

3. To determine the effects SMAD4 positive and negative cancer cells have on the expression of S100A8 and S100A9, using cell culture methodology and proteomic analysis with western blotting.

4. To determine potential cellular mediators of S100A8 and S100A9 induction in the pancreatic cancer microenvironment, using cell culture methodology and proteomic analysis with western blotting.

5. To produce recombinant GST tagged S100A8 and S100A9 protein and study their effects on pancreatic and colorectal cancer cell characteristics such as motility and proliferation. This would be undertaken using Boyden chamber experiments and MTT proliferation assay.

PART 2: METHODS, RESULTS AND DISCUSSION

CHAPTER EIGHT

IMMUNOHISTOCHEMISTRY METHODOLOGY AND ANALYSIS OF TISSUE MICROARRAY

8.1 Pancreatic Cancer Tissue Microarray

Pancreatic cancer tissue microarrays (TMAs) were generated at the Cancer Tissue Bank Research Centre, University of Liverpool, UK using the method described by Kononen *et al.* (Kononen J et al., 1998). Full ethical approval from the local research ethics committee along with patient's written consent for storage of tissue and use in research was obtained prior to construction of the microarray. The TMA contained matched sets of tumour and non-neoplastic pancreatic tissue samples from 71 pancreatic cancer patients treated at the Royal Liverpool University Hospital between 1994 and 2003.

The TMAs used contained pancreatic ductal adenocarcinoma tissue cores in duplicates from 71 patients along with matched normal tissue from 53 patients also in duplicates (a total of 248 cores). In addition, the array also contained 8 cores each of normal colonic, liver and kidney tissue samples for control purposes.

The TMAs were used to analyse the expression of S100A8, S100A9, CD68 and TGF-β1 in pancreatic cancer tissue. The proteins of interest were visualized on the TMAs with immunohistochemistry (IHC) and diaminobenzidine (DAB)-based detection (details given in 2.1.2). A consultant histopathologist, Dr Fiona Campbell and my supervisor Dr E Costello performed S100A8 and S100A9 expression analysis and scoring of the TMAs. All other staining and scoring (CD68 and TGF-β1) was undertaken by me under the

supervision of Dr Fiona Campbell and Mr Andrew Dodson (Department of Pathology, Royal Liverpool University Hospital).

The information recorded included the cell type stained (Tumour or Stroma), and the subcellular location of tissue staining (i.e. nuclear, cytoplasmic, both) [recorded for TGF-β1]. Intensity of staining (graded on a scale from 0 to 3, where 0 was no stain to blush and 3 was maximal brown staining) and the percentage of cells demonstrating positive immunoreactivity (0-100% of cells) was also recorded [recorded for TGF-β1]. In the case of CD68 (undertaken by me), S100A8 and S100A9 (undertaken by Dr E Costello), the exact number of positive cells per core were counted. For TGF-β1 staining the total score for each subcellular compartment was obtained as a product of the intensity and extent (percentage of cells stained) of staining.

Details of histopathological data for each case on the TMA were obtained from pathological reports. The data recorded included patient age, sex, tumour size and grade, lymph node and resection margin status and the presence or absence of perineural or vascular invasion. Survival status, and where applicable, the dates of death were obtained from a clinical database held by the pancreatic nurse specialist, general practitioners, cancer tissue bank registry and individual health authorities. The majority of the above-mentioned information was already present on the TMA database and any deficiencies were obtained and completed by myself.

8.2 Immunohistochemical staining (IHC)

Immunohistochemical staining was performed with the help of Mr Andrew Dodson for S100A8, S100A9 proteins (performed by Mr D Vimalachandran and myself), CD68 and TGF β-1 (performed by myself).

Stages used in Immunohistochemical staining

Slide preparation

Paraffin embedded pancreatic cancer tissue sections, five-micron in thickness were deparaffinised in xylene for 3 minutes and then rehydrated through decreasing alcohol concentrations (100%, 90%, 70%, 50%, 30%). Slides were placed in varying concentrations of alcohol for 15 seconds each and finally washed twice in distilled water. Peroxidises were blocked on the slides with a peroxidase block provided in the Dako Cytomation kit K4010 (Dako Systems), by incubation for 5 minutes. The slides were subsequently washed in tap and deionised water for 3 minutes each.

Antigen retrieval

Antigen retrieval was necessary for all primary antibodies (S100A8, S100A9, CD68, TGF-β1) used. This was performed by pressure-cooking the slides in a solution containing 10 mM EDTA (pH 7.4) for 3 minutes, followed by rapid cooling in tap water.

Staining with Primary antibodies

Immunohistochemical (IHC) staining was performed using either an automatic staining system (Autostainer, DAKO Cytomation) or manually. Primary antibody diluted up to 200μL in Chemomate, (protein buffered solution and detergent, DAKO Systems) was placed on each slide and incubated either 1 hour at room temperature or overnight at 4^0 C depending on when the experiment was conducted in the day.

Slides were incubated with either polyclonal goat anti-S100A8 antibody (Santa Cruz Biotechnology 1:200) or polyclonal rabbit anti-S100A9 antibody (Santa Cruz Biotechnology) 1:400) or polyclonal rabbit anti-TGF-β1 antibody (pre diluted, Abcam) or monoclonal mouse anti-CD68 antibody (1:400 Santa Cruz Biotechnology). Following incubation with primary antibody, the slides were washed thrice with Tris buffered saline (50 mM Tris, 150 mM NaCl, Adjusted to pH 7.6 with HCl) for 3 minutes on each occasion. Negative controls were incubated with the labelled secondary antibodies only.

Staining with Secondary Antibodies and Substrate for IHC

Antibody localisation was visualised by incubating sections with a horse radish peroxidase conjugated secondary antibody for 1 hour followed by 3 washes with tris buffered saline each lasting for 3 minutes. This step was followed by incubation with diaminobenzidine (DAB) (Dako systems) for 10 min. 20 μL of liquid DAB + Chromogen from DakoCytomation kit K4010 were mixed gently with 1 mL of buffer provided in the

kit to produce the substrate diaminobenzidine. Following incubation, the slides were washed thrice in Tris-buffered saline for 3 minutes each.

Haematoxylin staining

The slides were then counterstained with haematoxylin for 30 seconds, followed by a wash in Scott's water (1L tap water, 23mM sodium bicarbonate, 83mM $MgSO_4$) and then under normal tap water for 30 seconds each. The slides were dehydrated in varying concentration of ethanol (30%, 70%, and 100%) for 3 seconds each and then in 100% xylene for 3 seconds also. The slides were then air-dried and cover slips mounted with DPX mountant media (BDH compounds).

8.3 Immunofluorescence (IF) Staining

Immunofluorescent staining was performed under the supervision of Mr Andrew Dodson (Department of Pathology, Royal Liverpool University Hospital), in a similar manner to immunohistochemistry on paraffin embedded pancreatic cancer tissue slides. Stages of slide preparation and antigen retrieval were exactly the same for Immunofluorescence staining as they were for Immunohistochemical staining. Co-immunofluorescence was undertaken to determine whether S100A8 and S100A9 co-localised with other cellular markers. Slides were incubated with a variety of primary antibodies listed in Table 2.1, either singly or concurrently in pairs. Slides were then incubated in a dark room for 1 hour. Following incubation with primary antibodies the sections were washed in Tris-buffered saline.

Primary Antibody	Dilution
Polyclonal goat anti-S100A8	Santa Cruz Biotechnology diluted 1:200
Polyclonal rabbit anti-S100A9	Santa Cruz Biotechnology diluted 1:400
Polyclonal rabbit anti-TGF-β1	pre diluted, Abcam
Monoclonal mouse anti-CD68	Santa Cruz Biotechnology diluted 1:400
Monoclonal mouse anti-smooth muscle actin (smooth muscle / myofibroblast marker)	Santa Cruz Biotechnology diluted 1:50
Monoclonal mouse anti-desmin (smooth muscle / myofibroblast marker)	Santa Cruz Biotechnology diluted 1:40
Polyclonal goat anti-vimentin	Santa Cruz Biotechnology diluted 1:200
Monoclonal mouse anti-CD20 (B lymphocyte marker)	Santa Cruz Biotechnology diluted 1;4000
Monoclonal mouse anti-CD34 (endothelial marker)	Santa Cruz Biotechnology diluted 1:50
Monoclonal mouse anti-CD38 (plasma cell marker)	Santa Cruz Biotechnology diluted 1:100
Monoclonal mouse anti-CD3 (T-cell marker)	Santa Cruz Biotechnology diluted 1:100
Monoclonal mouse anti-CD79A (B-cell marker)	Santa Cruz Biotechnology diluted 1:500
Monoclonal mouse anti-mast cell trypsin (mast cell marker)	Santa Cruz Biotechnology diluted 1:400

Table 2.1: Table enumerating various primary antibodies and their concentration used.

Staining with Secondary Antibodies for Immunofluorescence

Detection of bound antibody was achieved by manual staining with FITC-labelled donkey anti-goat IgG (30μg/mL, Santa Cruz Biotechnology), TRITC-labelled swine anti-rabbit IgG (25μg/mL DakoCytomation), FITC- labelled conjugated horse anti-mouse IgG (Vector Laboratories, 1:50) fluorescently labelled secondary antibodies on a flat-bed incubation tray in a dark room. Incubation was undertaken for 60 minutes with a mixture of either one or two antibodies.

Mounting

Sections were washed in Tris-buffered saline thrice for 3 minute each and mounted in aqueous mounting medium containing DAPI 4, 6-diamidino-2-phenylindole (Vector Laboratories, H-1500).

Microscopy and photography

Microscopy and photography of co-immunofluorescence stained sections was undertaken immediately, once mounting was complete, on a fluorescence microscope to achieve sharp image quality free of background noise.

8.4 Co- immunofluorescence analysis

In order to identify the cells expressing S100A8 and S100A9, co-immunofluorescent analysis was undertaken. To analyse whether S100A8 and S100A9 are co-expressed, immunofluorescent double staining experiments were performed (n=5) on independent pancreatic cancer cases. Serial sections were incubated separately or concurrently with polyclonal goat anti-S100A8 and / or polyclonal rabbit anti-S100A9 antibody. In all cases, this was followed by the application of two fluorescently labelled secondary antibodies: FITC-labelled donkey anti-goat IgG for the detection of the S100A8 antibody and the TRITC-labelled swine anti-rabbit IgG for the detection of S100A9 antibody. As demonstrated in Figure 2.1(I) and 2.1(II), under conditions of single labelling S100A8 immunoreactive cells are green and S100A9 immunoreactive cells are red. Cross-reactivity between primary goat and secondary rabbit or primary rabbit and secondary goat antibodies was not detected and the tumour cells remained unstained for either S100A8 or S100A9 protein as demonstrated by their blue colour.

Dual staining (Figure 2.1(III) and (IV)) revealed the presence of S100A8 and S100A9 in the same cells presenting as yellow. Under the same conditions (of dual staining) a number of cells showed expression of S100A9 only (red), (photographed more clearly in Figure 2.1 (III)) whereas no cells expressed S100A8 only (green). Therefore, S100A8 was detected in a subset of S100A9-positive cells contained within the stromal compartment of pancreatic cancer sections.

Morphologically S100A8- and S100A9-positive cells resembled cells of myeloid linage, possibly monocytes/ immature macrophages. To further evaluate the nature of the S100A8 and S100A9 expressing cells, immunofluorescent double staining experiments on pancreatic cancer specimens were undertaken with a variety of cellular markers. These experiments showed no co-localisation with CD68, a marker for mature macrophages (Figure 2.2 (I)), and positive co-localisation of S100A9 with monocytic marker CD14 (Figure 2.2 (II)).

The co-expression of S100A9 was also evaluated with other cell types, which form part of the pancreatic cancer's dense stroma. This was undertaken using immunofluorescent double staining with a variety of cellular markers, including markers for T-cell (Figure 2.3(I)), fibroblasts (Figure 2.3(II)), B-lymphocytes (Figure 2.3 (III), myofibroblasts (Figure 2.3 (IV)), plasma cells (Figure 2.3 (V)), endothelial cells (Figure 2.3 (VI)), mast cells (Figure 2.3 (VII)), and B-cell marker (Figure 2.3 (VIII)). Co-expression of S100A9 was not detected with any of these cellular markers as seen in the Figure 2.3.

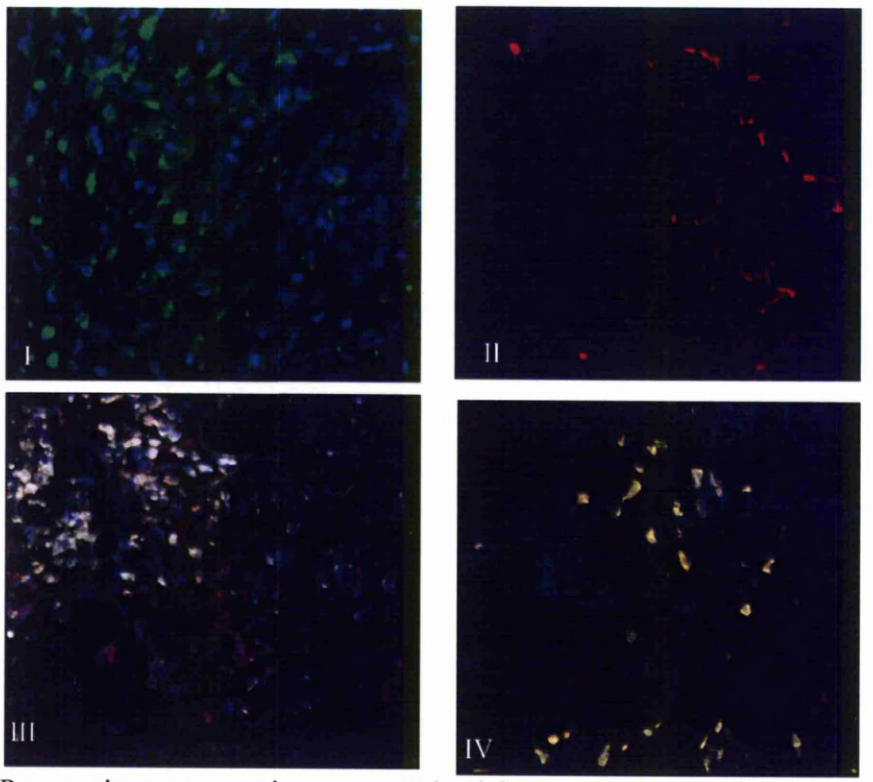


Figure 2.1: Pancreatic cancer sections were stained for (I) immunofluorescence analysis with anti-S100A8-FITC (green), (II) anti-S100A9-TRITC (red), (III) co-stained with anti-S100A8-FITC and anti-S100A9-TRITC at low magnification X20 (IV) co-stained with anti-S100A8-FITC and anti-S100A9-TRITC at high magnification X40.

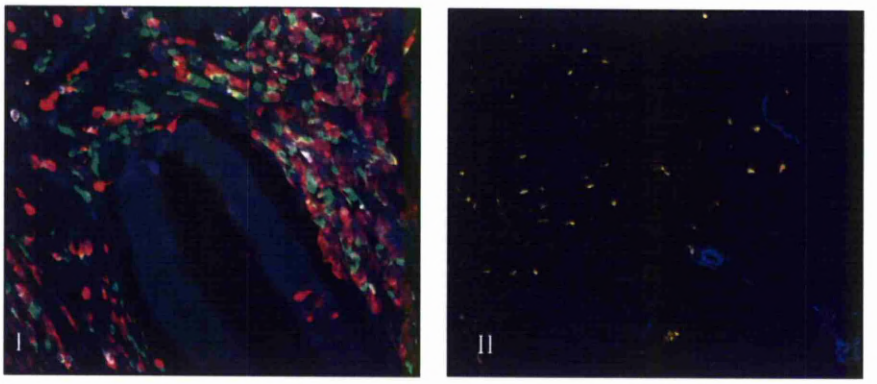


Figure 2.2: Pancreatic cancer sections showing co-staining of anti-S100A9-TRITC (red) with (I) anti-CD68-FITC (green), (II) anti-CD14-FITC (yellow)

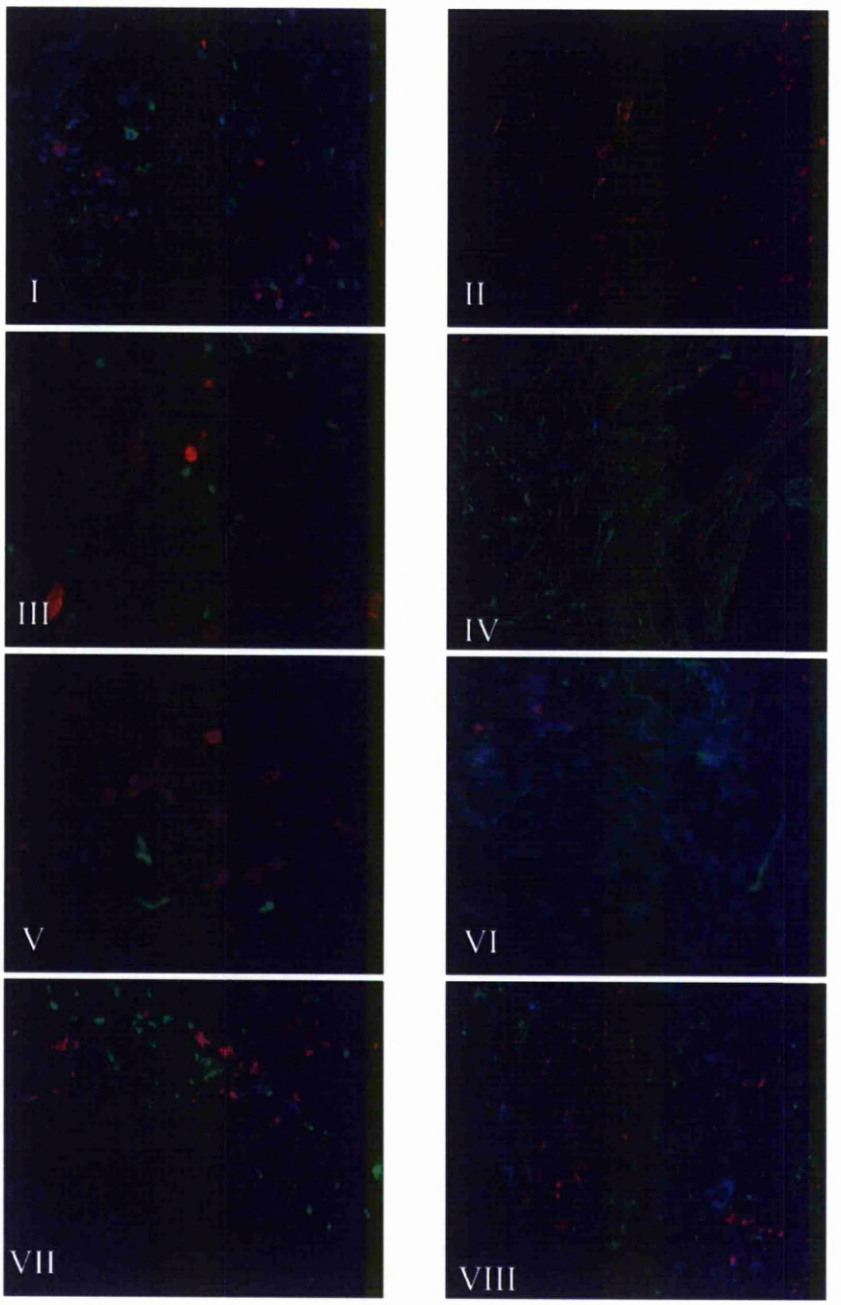


Figure 2.3: Pancreatic cancer sections showing co-staining of anti-S100A9-TRITC (red) with (I) Anti-CD3-FITC (green), (II) Anti-vimentin-FITC (green), (III) Anti-CD20 - FITC (green), (IV) Anti-smooth muscle actin-FITC (green), (V) Anti-CD38-FITC (green), (VI) Anti-CD34-FITC (green), (VII) Anti-mast cell trypsin--FITC (green), (VIII) Anti-CD79A--FITC (green)

8.5 Association of stromal S100A8 and S100A9 expression with TGF-β1 and CD68 expression in pancreatic cancer tissue

Findings of mouse model experiments by Hiratsuka *et al.* suggested the role of S100A8 and S100A9 in cancer metastasis (Hiratsuka et al., 2006). They showed that secreted factors from the primary tumours (i.e. TGF-β, TGF-α and VEGF-A) induced expression of S100A8 and S100A9 in myeloid cells in lung tissue, in turn aiding metastases of primary tumour cells to the lung. Therefore, the expression of TGF-β1 in the pancreatic cancer tissue (ductal and stromal elements) and its association with stromal S100A8 and S100A9 expression was examined. The lack of overlap in expression between S100A8/S100A9 and CD68 on the co-immunoflorescence, prompted me to examine the relationship between S100A8/S100A9 expressing cells and CD68 expressing cells in pancreatic cancer tissue.

TGF-β1 expression in the pancreatic cancer tissue and its association with stromal S100A8 and S100A9 expression

Immunohistochemical analysis of pancreatic cancer TMAs, revealed differential levels of TGF-β1expression in the nucleus (nuclear TGF-β1, nTGF-β) and cytoplasm (cytoplasmic TGF-β1, cTGF-β) of pancreatic cancer cells and its surrounding stroma (stromal TGF-β1, sTGF-β) (Figure 2.4). Differential expression of TGF-β1 in stromal cytoplasm and nucleus was not possible owing to uniform staining in terms of intensity and pattern in both these compartments.

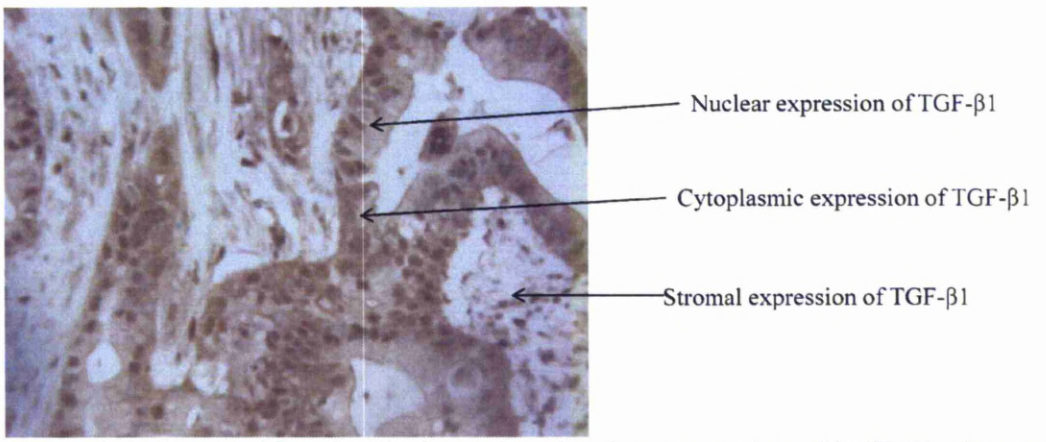


Figure 2.4: Immunohistochemical staining showing expression of TGF-β1 observed in the nucleus (nTGF-β1) and cytoplasm (cTGF-β1) of pancreatic cancer cells and surrounding stroma (sTGF-β1).

For the purpose of examining associations between tumour (nuclear and cytoplasmic) and stromal TGF-β1 expression the patients were categorised into two groups, i.e. those having low levels of expression with immunohistochemical scores of less than or equal to the median and those with high levels of expression i.e. having a score greater than the median score. All patients (n=69) expressed the presence of TGF-β1 in the tumour (nucleus and cytoplasm) and in stromal cells. The median immunohistochemical score for the 3 compartments was nTGF-β1: 80 (IQR: 25-147.8), cTGF-β1: 200 (IQR 140-200), and sTGF-β1: 90 (IQR: 50-140) (Table 2.2).

COMPARTMENT EXPRESSING TGF-β1	TOTAL PATIENTS	LOW LEVELS OF EXPRESSION n (%)	HIGH LEVELS OF EXPRESSION n (%)
Nuclear TGF-β1 (nTGF-β1)	69	38 (55.1%)	31 (44.9%)
Cytoplasmic TGF-β1 (cTGF-β1)	69	16 (13.2%)	53 (76.8%)
Stromal TGF-β1 (sTGF-β1)	69	25 (36.2%)	44 (63.8%)

Table 2.2: Tables demonstrating numbers of patients expressing high and low levels of TGF-β1expression in various compartments.

High levels of tumour nuclear TGF-β1 expression were significantly associated with high stromal TGF-β1 expression ($p = 0.04$; Mann Whitney test). Interestingly, tumour nuclear nTGF-β1 expression was not associated with tumour cytoplasmic TGF-β1 levels ($p=0.09$; Mann Whitney test). In addition, tumour cytoplasmic expression of TGF-β1 showed no correlation to stromal TGF-β1 expression either ($p=0.068$; Mann Whitney test).

Based on the information held in the pancreatic cancer database nuclear, cytoplasmic and stromal TGF-β1 expression in pancreatic cancer tissue was correlated to patient clinco-pathological parameters. Expression in these compartments was correlated to patient age, gender, size of tumour, differentiation grade, vascular invasion, perineural invasion and nodal metastasis, however no significant associations (Fishers exact test) were established (Table 2.3).

PATIENT PARAMETERS	NUCLEAR TGF-β1 EXPRESSION (n=69)			CYTOPLASMIC TGF-β1 EXPRESSION (n=69)			STROMAL TGF-β1 EXPRESSION (n=69)		
	High (n)	**Low (n)**	**P-Value**	**High (n)**	**Low (n)**	**P-Value**	**High (n)**	**Low (n)**	**P-Value**
Age									
<60yrs	9	11	0.9	15	5	0.9	14	6	0.6
>60yrs	22	27	(ns)	37	12	(ns)	30	19	(ns)
Sex									
Male	19	22	0.8	30	12	0.6	23	18	0.2
Female	12	16	(ns)	21	6	(ns)	20	8	(ns)
Size of tumor									
< 20mm	7	14	0.3	15	6	0.9	14	7	0.8
> 20mm	24	24	(ns)	36	12	(ns)	29	19	(ns)
Differentiation grade									
Well-moderate	17	28	0.1	32	11	0.8	32	12	0.4
Poor	11	13	(ns)	19	7	(ns)	11	14	(ns)
Vascular invasion									
Yes	26	28	0.5	39	14	0.9	32	21	0.3
No	3	6	(ns)	8	2	(ns)	8	2	(ns)
Unrecorded	3	3		2	4		4	2	
Perineural invasion									
Yes	28	35	0.9	44	16	0.6	39	24	0.3
No	1	2	(ns)	5	1	(ns)	3	0	(ns)
Unrecorded	1	2		0	3		0	3	
Nodal metastasis									
Yes	26	30	0.8	41	13	0.9	33	22	0.3
No	5	8	(ns)	11	4	(ns)	11	3	(ns)

Table 2.3: Pancreatic cancer TGF-β1 expression in tumour nuclear, tumour cytoplasm and stromal compartments and its association to clinicopathological parameters.

The correlation between TGF-β1 expression in pancreatic cancer (tumour nuclear, tumour cytoplasm and stroma) and the number of S100A8 and S100A9 expressing cells was also determined as established previously. Tumour nuclear TGF-β1 expression significantly correlated to the number of stromal S100A8 expressing cells ($p = 0.0488$; Mann Whitney test) as well as the number of S100A9 expressing cells ($p = 0.0138$; Mann Whitney test) (Figure 2.5). On the other hand, tumour cytoplasmic TGF-β1 significantly correlated with the number of cells expressing S100A8 ($p = 0.0374$; Mann Whitney test) only and not S100A9 ($p = 0.115$; Mann Whitney test) (Figure 2.5). Stromal TGF-β1 showed no significant correlation with the number of cells expressing both S100A8 ($p = 0.1377$; Mann Whitney test) and S100A9 ($p = 0.1377$; Mann Whitney test) (Figure 2.5).

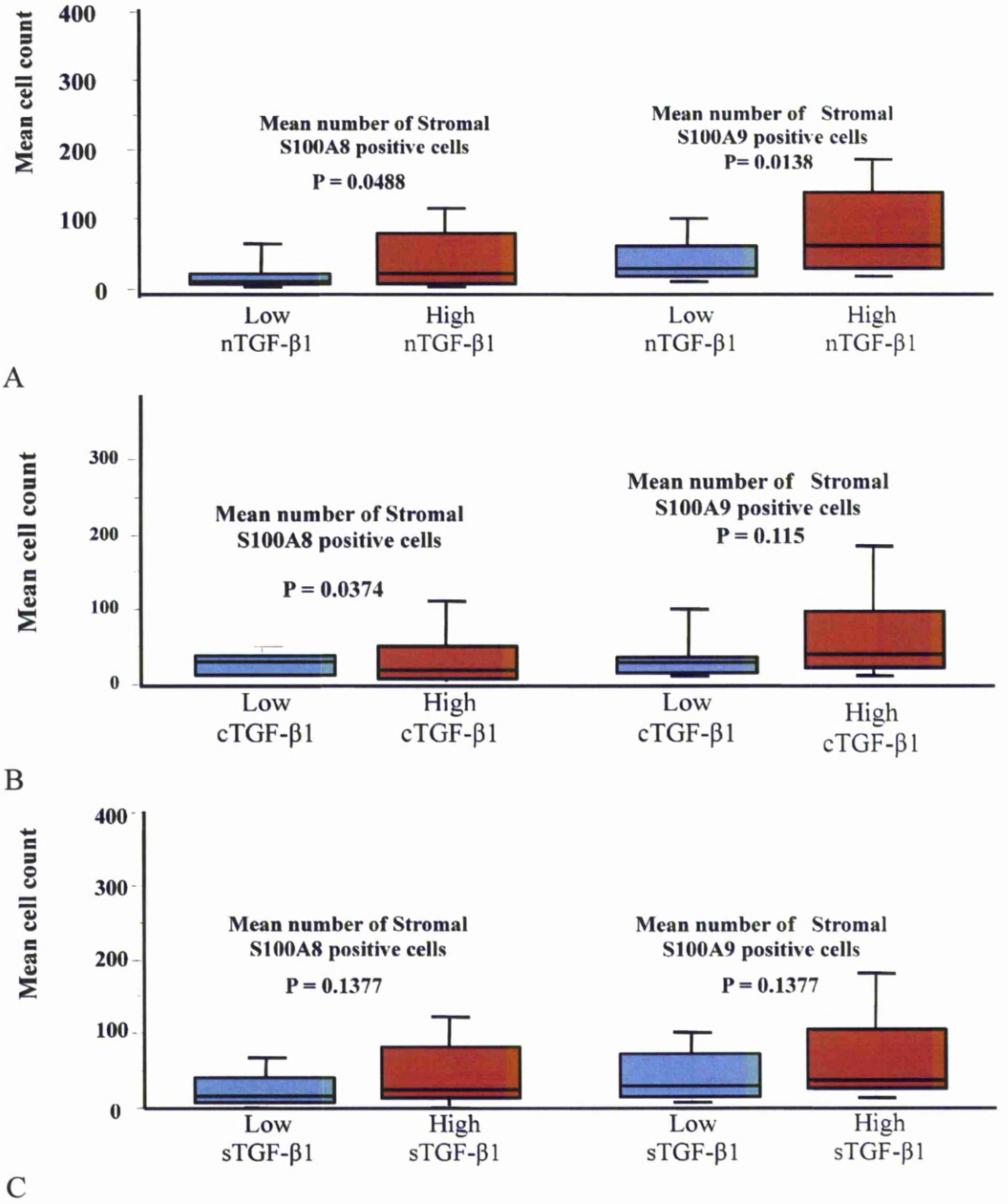


Figure 2.5: Figure demonstrating the association of (A) tumour nuclear (nTGF-β1), (B) tumour cytoplasmic (cTGF-β1) and (C) stromal (sTGF-β1) and mean numbers of S100A8- and S100A9- positive stromal cells.

Survival analysis using a Kaplan Meier curve (Figure 2.6) assessing the impact of nTGF-β1, cTGF-β1 and sTGF-β1 expression on patient survival found patients with high nTGF-β1 expression in the pancreatic cancer had a significantly poorer 2-year survival compared to those with lower levels of expression ($p = 0.04$). Univariate and multivariate Cox proportional hazards regression analyses was used to predict survival for all clinico-pathologic parameters (patient age, gender, size of tumour, differentiation grade, vascular invasion, perineural invasion and nodal metastasis) and nTGF-β1 expression, however none were significant (Table 2.4). Only nTGF-β1 expression remained an independent predictor of survival in the multivariate analysis with hazard ratio of 0.5 (0.3 – 0.9, $p = 0.03$).

Variables	Categories	Cases (n = 69)	Univariate analysis		Multivariate analysis	
			HR (95% CI)	P-value	HR (95% CI)	P- value
Age	<60yrs >60yrs	20 49	0.6 (0.3 – 1.3)	**0.2**	0.6 (0.3 – 1.2)	0.1
Gender	Female Male	28 41	0.9 (0.5–1.6)	0.8		
Nodal metastasis	No Yes	13 56	0.6 (0.3 – 1.4	0.3	0.8 (0.3 – 1.5)	0.3
Differentiation grade	Well- moderate Poor	 45 24	0.8 (0.4 – 1.4)	0.3		
Size of tumour	< 20mm > 20mm	21 48	0.6 (0.3 – 1.1)	**0.09**	0.6 (0.3 – 1.3)	0.2
Vascular invasion	No Yes unrecorded	9 54 6	0.8 (0.4 –1.8)	0.6		
Perineural invasion	No Yes unrecorded	3 63 3	0.7 (0.2 – 2.9)	0.6		
Mean Stromal S100A8 cells	-	69	1 (0.9 – 1)	0.2	1 (1– 1.1)	0.6
Mean Stromal S100A9 cells	-	69	1(0.9 – 1)	0.2	1 (1– 1.1)	0.9
Nuclear TGF-β1	**Low** **High**	**38** **31**	**0.6(0.4–1)**	**0.04**	**0.5 (0.3 – 0.9)**	**0.028**

Table 2.4: Univariate and multivariate Cox proportional hazards regression analyses to derive risk estimates related to survival for all clinicopathological parameters and nTGF-β1 expression.

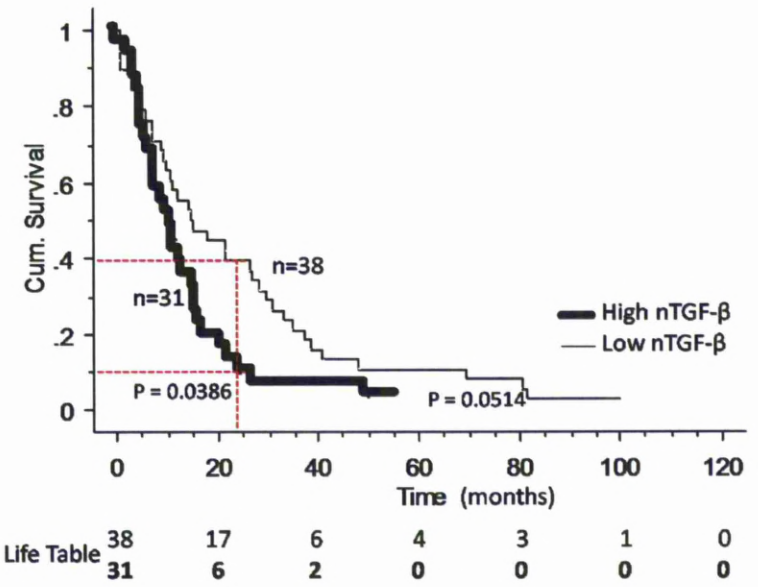


Figure 2.6: Kaplan Meier curve showing the difference in 2-year survival for nTGF-β1 expression.

Pancreatic cancer stromal CD68 expression and its association with S100A8 and S100A9 protein expression

The presence of CD68 expressing macrophages (marker for tissue macrophages) was also found to be present in the pancreatic cancer associated stroma as previously been reported in the literature (Figure 2.7).

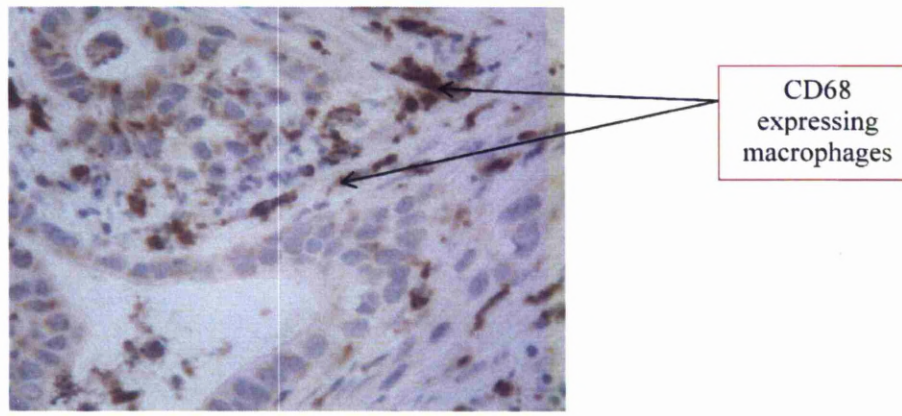


Figure 2.7: Figure demonstrating the presence of CD68 positive macrophages in the pancreatic cancer stroma.

For the purpose of examining associations between the number of CD68 immunopositive cells and other patient parameters, patients were categorized into two groups, i.e. those having CD68-positive cell numbers less than or equal to the median (low levels of CD68 expression) or those having CD68-positive cells greater than the median (high levels of CD68 expression). Similarly, for examining associations for S100A8- or S100A9-immunopositive cells the patients were categorised in a similar manner i.e. into two groups, those having positive cell numbers less than or equal to the median or greater than the median for the two respective S100 proteins.

The number of positively immunostained cells in each tumour core of the pancreatic cancer TMA were counted for the presence of CD68 positive cells. All patients (n=71) expressed the presence of CD68 positive cells in the tumour stoma. The mean number of cells per duplicate core was 96.7 (range 24-275 cells) with the median being 83.5 (IQR 63.5). Thirty-five patients (49.2%) had low levels of CD68 positive cells, compared to 36 (50.8%) patients who demonstrated high levels of CD68 positive cell expression in the pancreatic cancer stroma. Tumours containing low numbers of CD68-positive cells correlated significantly with the tumours which had low numbers of S100A8- positive cells (p=0.001; Fisher's exact test) and S100A9-positive (p=0.0001; Fisher's exact test) cells (Figure 2.8).

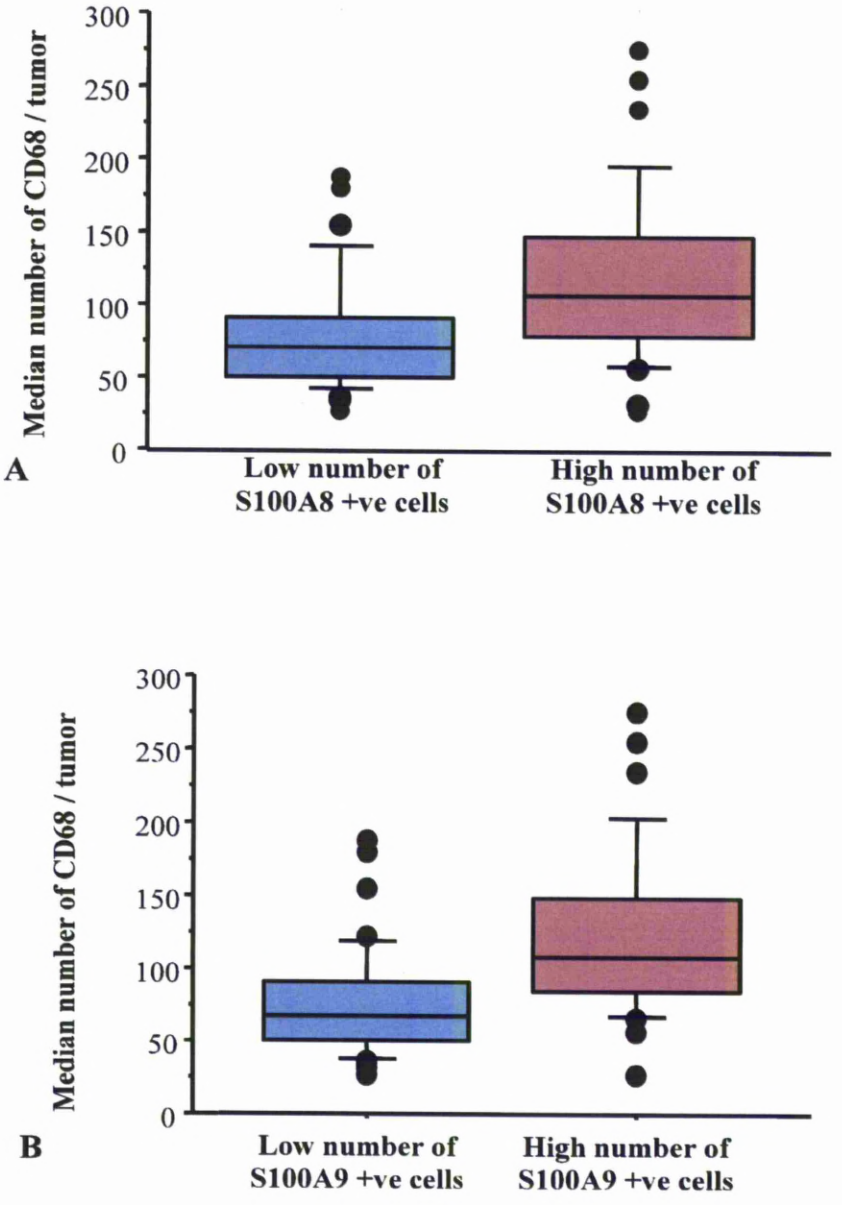


Figure 2.8: Box plots demonstrating the association of CD68 positive cells and S100A8 positive (A) and S100A9 positive (B) cells in the pancreatic cancer stroma.

Using the information held in the pancreatic cancer database the number of CD68-positive cells in the pancreatic cancer stroma was correlated to patients clinico-pathological parameters, which included age, gender, size of tumour, differentiation grade, vascular invasion, perineural invasion and nodal metastasis. No significant association (Fishers exact test) was established between the number of CD68 positive cells and these patient parameters (Table 2.5). Moreover, the number of CD68- positive cells did not confer any survival benefit (Survival analysis using a Kaplan Meier: p=0.4) (Figure 2.9).

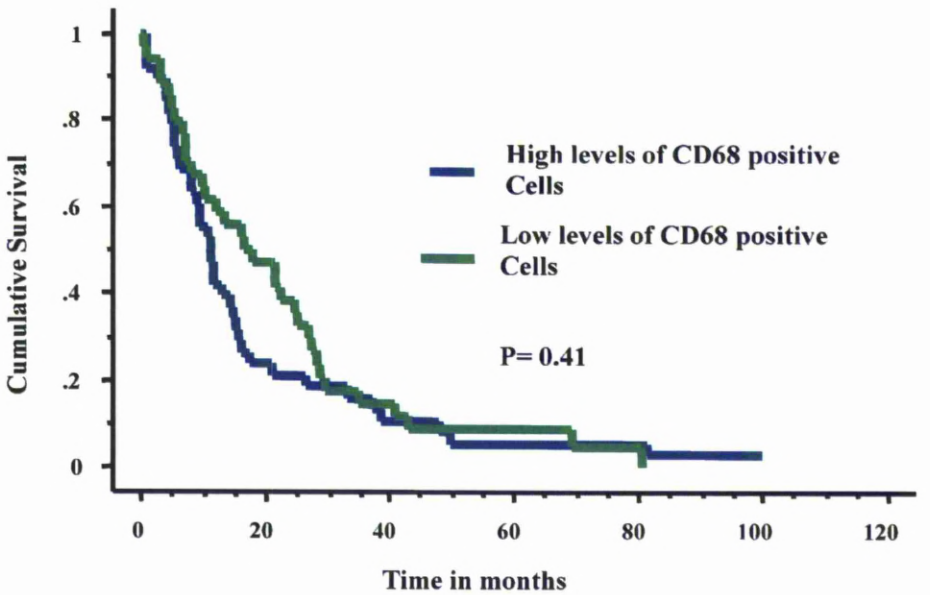


Figure 2.9: Kaplan Meier curve showing the difference in survival for low and high levels of CD68 positive cells in the pancreatic cancer stroma.

Parameter	All Cases n=71	Low $CD68^+$ (≤ median) n=35	High $CD68^+$ (> median) n=36	P-value
Age				
<60yrs	21	5	16	0.1
>60yrs	50	29	21	
Sex				
Male	43	20	23	0.8
Female	28	14	14	
Size of tumor				
< 20mm	22	13	9	0.2
> 20mm	49	21	28	
Differentiation grade				
Well-moderate	46	20	26	0.2
Poor	25	14	11	
Vascular invasion				
Yes	54	27	27	0.5
No	11	4	7	
Unrecorded	6	4	2	
Perineural invasion				
Yes	65	31	34	0.9
No	3	1	2	
Unrecorded	6	3	3	
Nodal metastasis				
Yes	57	26	31	0.6
No	14	8	6	

Table 2.5: Pancreatic cancer stromal CD68 expression and its association to clinicopathological parameters

8.6 Discussion

Based on the proteomic analysis of microdissected stromal and ductal elements of pancreatic cancer tissue undertaken by Mr D Vimalachandran it was determined that S100A8 and S100A9 proteins are expressed in the stromal cellular compartment. Moreover, the immunohistochemical analysis undertaken by Dr Costello confirmed the presence of cells expressing both S100A8 and S100A9 in the stroma and not in the malignant epithelial elements.

I undertook a detailed review of the literature to understand the relationship these proteins had in the in the tumour microenvironment. The literature indicated the presence of S100A8 and S100A9 to be linked to monocytes and immature macrophages (Nacken et al., 2003; Odink et al., 1987) being implicated in a number of inflammatory disorders (Goebeler et al., 1994); (Gebhardt et al., 2006; Nacken et al., 2003; Srikrishna, 2012).

Immunofluorescent co-localisation experiments on pancreatic cancer specimens showed co-localisation of S100A9 with CD14, a monocyte/ immature macrophage marker, confirming the expression of these proteins in monocytes as indicated in the literature (Nacken et al., 2003). Additionally, expression of S100A8 was detectable in a subset of S100A9-positive cells. Neither of the S100 proteins co-expressed with CD68, a marker of mature tissue macrophages (Lewis and Pollard, 2006; Ruffell et al., 2012). Moreover, the cellular markers of smooth muscle, myofibroblasts, lymphocytes, T- and B-cells, endothelial cells, mast cells and plasma cells also failed to co-localise with S100A9. The presence of Tie2 was not assessed on the TMA as TEMs in humans express CD14, CD16

and CD11c surface markers and are similar to the so-called "resident monocytes" in morphological appearances (Gordon and Taylor, 2005; Kurahara et al., 2009). There is no association in the literature linking expression of S100A8 and S100A9 to TEMs, with TEMs involvement in carcinogenesis not fully understood. Immunofluorescent co-localisation findings confirmed the presence of these proteins to be in monocytes which was consistent with the reported abundant expression of these proteins in cells of the myeloid lineage (Nacken et al., 2003; Srikrishna, 2012) . Zwadlo *et al.* demonstrated the presence of both S100 proteins in monocytes which were expressed specifically at early stages of monocyte differentiation being down regulated during maturation to macrophages (Zwadlo et al., 1988). This is believed to change as monocytes, recruited from the blood stream to sites of inflammation differentiate to mature macrophages. They initially express both S100A8 and S100A9 and as they mature S100A8 expression ceases leaving only S100A9 which is also subsequently lost as the cell matures further (Zwadlo et al., 1988). The co-immunofluorescence data is consistent with the presence of S100A8 in a subset of S100A9-positive pancreatic cancer stromal cells. Our findings of the immunohistochemical analysis of the TMA, SMAD4 negative tumours demonstrated significantly fewer number of S100A8-postive cells in the stroma compared to S100A9, moreover the ratio of S100A9/S100A8 was significantly higher in SMAD4 negative tumour compared to SMAD4 positive tumours (Sheikh et al., 2007). Thus, a strongly negative relationship between the expression of SMAD4 in tumours cells and the expression of S100A8 in stromal inflammatory cells was established. It is thus possible that the maturation process of monocytes to macrophages in the pancreatic cancer tumour microenvironment is influenced by the SMAD4 status of tumour. The precise function of

S100A8 and S100A9 in the pancreatic cancer tumour microenvironment was unclear, although their expression and secretion are likely to contribute to the host inflammatory response to the tumour.

Monocytes and macrophages are both haematopoietic cells, which are derived from common CD34-positive progenitor cells, being produced in the bone marrow (Kurahara et al., 2009; Murdoch et al., 2008). In cancer these cells have a major role being involved in all aspects of tumour biology, namely invasion recruitment of leukocytes, activation of T-cells, angiogenesis, growth and metastasis hence making them an essential regulator in the tumour microenvironment (Coffelt et al., 2009; Joyce and Pollard, 2009; Lamagna et al., 2006; Schmid and Varner, 2007). Tissue associated macrophages (TAMs) have gained significant importance for their role in carcinogenesis in a variety of cancers being highly versatile and multifunctional components of the innate immune system (Kurahara et al., 2012a; Lamagna et al., 2006; Ruffell et al., 2012). Owing to the presence of high levels of monocytes in the microenvironment of pancreatic cancer, I undertook an immunohistochemical analysis of the pancreatic cancer TMA analysing the presence of CD68 positive cells in the stroma. Tissue macrophages express a variety of surface markers such as CD163 and CD204 with CD68 being well established as a marker for TAMs (Pollard, 2004). My findings were consistent with the literature indicating the presence of CD68 positive macrophages in the pancreatic cancer stroma, however no associations between the number of CD68 immunopositive cells and patient clinco-pathological parameters, in particular survival was established (Kurahara et al., 2009). This is consistent with the findings of Kurahara *et al.*, who have shown that TAMs

expressing CD163 and CD204, conferred a worse prognosis exhibiting increase lymphatic metastasis in pancreatic cancer. This was not the case with CD68 expression (Kurahara et al., 2009). The same author has very recently demonstrated the presence of folate receptor expressing TAMs in pancreatic cancers, which confer a high incidence of haematogenous metastasis and poor survival (Kurahara et al., 2012b). The number of CD68-positive cells in the tumour correlated significantly with the S100A8- positive and S100A9-positive cells indicating a common precursor cell, being that of myeloid linage.

S100A8 and S100A9 have been associated with a sentinel role in cancer metastasis (Hiratsuka et al., 2006) particularly in the development of the "pre-metastatic niche". It has been demonstrated in a mouse model experiment that S100A8 and S100A9 expression was induced in myeloid and endothelial cells in the lungs tissue of mice by secreted soluble factors such as TGF-β, TGF-α and VEGF-A. These were derived from the distant primary cancer cells. The presence of S100A8 and S100A9 in the lung tissue primed the microenvironment in what has been termed the premetastic phase hence aiding migration and implantation of the tumour cells. The study from Hiratsuka *et al.* indicated the role of S100A8 and S100A9 in the carcinogenesis process in a lung model, which suggested a similar process could likely be taking place in the pancreatic cancer microenvironment. Similar findings described in the more recent literature revolving around myeloid-derived suppressor cells (MDSCs) and the presence of S100A9 protein, show the presence of S100A9 enhances the production of MDSC in tumours (Cheng et al., 2008).

The functions of monocytes / macrophages in the cancer microenvironment are numerous with the presence of TGF-β being central to a variety of functions (Matthaios et al., 2011; Tjomsland et al., 2011) (Srikrishna, 2012). My immunohistochemical analysis of the pancreatic cancer TMA findings indicated an association in the pancreatic cancer microenvironment between stromal and tumour nuclear TGF-β1 expression; however no correlation was seen with tumour cytoplasmic TGF-β1 levels. More importantly, however tumour nuclear TGF-β1 expression significantly correlated to stromal S100A8 and S100A9 expression with no correlation seen with stromal TGF-β1 expression. This indicated the possible role of tumour TGF-β1 in influencing expression and in turn the maturation of monocytes in the pancreatic cancer stroma. This is a plausible association, bearing in mind the findings of Hiratsuka *et al.* who have demonstrated the presence of secreted soluble factors (TGF-β, TGF-α and VEGF-A) as drivers of S100A8 and S100A9 expression in lung tissue of mouse models (Hiratsuka et al., 2006). TGF-β1 expression is overexpressed in pancreatic cancer tissue with increased expression of all three isoforms noted (Friess et al., 1993). This overexpression is associated with advanced stage and poor survival (Friess et al., 1993). My findings were consistent with the literature suggesting high TGF-β1 overexpression being associated with a poor 2-year survival compared to those with lower levels of expression. This is primarily attributed in studies due to a loss of TGF-β1's tumour suppressive and growth inhibitory effects (Truty and Urrutia, 2007b).

Based on the findings of Hiratsuka *et al.*, as well as our observation regarding the association of S100A8 and S100A9 expression in pancreatic cancer and SMAD4

expression (Sheikh et al., 2007), I devised experimentation to future elucidate the influence conditioned media from pancreatic cancer had on the expression of S100A8 and S100A9 on monocytic cell lines and human monocytes. Moreover, a significant association between tumour nuclear TGF-β1 expression and S100A8 and S100A9 expression in stromal monocytes suggested crosstalk in the pancreatic cancer microenvironment.

CHAPTER NINE

CELLULAR EXPERIMENTS AND PROTEOMIC ANALYSIS

9.1 Cancer cell lines used in these experiments

Cell lines are invaluable tools for in-vitro investigation of tumour biology and genetics. There are approximately 40 different cell lines for pancreatic ductal adenocarcinoma, over 50 different colorectal and 7 human monocytic cell lines which are widely in use (the American tissue type culture collection, ATCC). Cell lines are quite heterogeneous as reflected by their grade of differentiation and diversity in structure and function. Data derived from cell lines should be interpreted in the background of their morphological differentiation, functional properties, growth kinetics and main genetic changes. Cell lines acquire additional genetic mutation during the process of their manipulation, therefore assessment of genes and proteins being studies should be accessed at all stages of the experiments.

Pancreatic cancer cell lines

Four pancreatic cancer cell lines (PANC-1, SUIT-2, BxPC3, and CFPAC-1) were utilized to provide conditioned media. In addition, motility and proliferation of PANC-1, SUIT-2 and MIA PaCa-2 pancreatic cancer cell lines was also studied when they were treated in vitro with GST tagged recombinant S100A8 and S100A9. These cell lines were derived from the liquid nitrogen storage facility of the Division of Surgery and Oncology, University of Liverpool. Pancreatic cancer cell lines have been analysed for alteration in K-*RAS*, p53 $p16^{INK4A}$ and SMAD4 genes (Moore et al., 2001; Sipos et al., 2003), which are summarized in the table below (Table 2.6). Based on their diverse variability in their molecular alterations particularly SMAD4 status, these cells were chosen. Their ability to express SMAD4 protein was subsequently analysed using western blotting of cell lysate.

Four pancreatic cancer cell lines (PANC-1, SUIT-2, BxPC3, and CFPAC-1) were utilized to provide conditioned media. For motility and proliferation experiments PANC-1, SUIT-2 and MIA PaCa-2 pancreatic cancer cell lines were studied following treatment with in vitro with GST-tagged recombinant S100A8 and S100A9. These cell lines were derived from the liquid nitrogen storage facility of the Division of Surgery and Oncology, University of Liverpool. These were chosen for study as Dr E Costello's group had utilized these cell lines in the past for study of proliferation and motility assays (Thompson et al., 2007)

LL IE	KRAS		p53		p16		SMAD4/DPC	
	Alteration	**Predicted Product**	**Alteration**	**Predicted Product**	**Alteration**	**Predicted Product**	**Alteration**	**Predicted Product**
PC3-3	None	Wild type	220 TAT-TGT	Tyr to Cys	Heterozygous deletion	Absent	Heterozygous deletion	Absent
PAC-1	12 GGT-GTT	Gly to Val	242 TGC-CGC	Cys to Arg	Methylated	Absent	Heterozygous deletion	Absent
NC-1	12 GGT-GAT	Gly to asp	273 CGT-CAT	Arg to His	Heterozygous deletion	Absent	None	Wild Type
IT-2	12 GGT-GAT	Gly to Asp	273 CGT-CAT	Arg to His	69 GAG-TAG	Glu to stop	None	Wild Type
A Ca-2	12GGT-TGT	Gly to Cys	248 CGG-TGG	Arg to Trp	Heterozygous deletion	Absent	None	Wt

Table 2.6: Table showing *KRAS, p53, p16* and *SMAD4* mutation in 5 pancreatic cancer cell lines

BxPC-3

This primary human pancreatic cancer cell line (Tan et al., 1986) was established from a biopsy specimen of a histologically confirmed adenocarcinoma of the body of the pancreas in a 61 years old female in 1986. Histopathologically, the tumours grown in nude mice exhibited the original characteristics of the primary adenocarcinoma. These features included the production of traceable mucin in moderately well to poorly differentiated adenocarcinomas with occasional lymphocytic infiltrations at the tumour peripheries (Tan et al., 1986).

CFPAC-1

This cancer cell line (Schoumacher et al., 1990) was derived from a pancreatic ductal adenocarcinoma (liver metastasis) of a patient with cystic fibrosis. The cells exhibit ion transport deficiencies consistent with cystic fibrosis, expressing the product of the CF gene. These cells manifest the most common CF mutation, deletion of three nucleotides resulting in a phenylalanine-508 deletion. CFPAC-1 cells show epithelial morphology and express cytokeratin and oncofoetal antigens characteristic of pancreatic duct cells.

PANC-1

This is an epithelioid cell line (Lieber et al., 1975), started from a human pancreatic carcinoma of ductal cell origin. Chromosome studies show a modal number of 63 with three distinct marker chromosomes and a small ring chromosome. The cells possess the type B phenotype for glucose-6-phosphate dehydrogenase (G6PD) and over expresses

human epidermal growth-factor receptor-2 (HER2/neu) oncogene but are oestrogen receptor (ER) negative.

SUIT-2

SUIT-2 cell lines (Iwamura et al., 1987) are derived from a metastatic liver tumour of human pancreatic carcinoma in 1987. These cells histopathologically closely resemble the original neoplasm, which was a moderately differentiated tubular adenocarcinoma. The SUIT-2 cell line produces and releases at least two tumour markers, carcinoembryonic antigen and carbohydrate antigen 19-9. It propagates well even in serum-free medium, and metastasizes to the regional lymph nodes in nude mice xenografts.

MIA PaCa-2

The MIA PaCa-2 cell line was established by A. Yunis *et al.* in 1975 from pancreatic cancer tissue procured form a 65-year-old Caucasian male. This is a hypotriploid human cell line with a few normal chromosomes absent. The cell line has a rapid doubling time of about 40 hours and a colony-forming efficiency in soft agar (Yunis et al., 1977)

Colorectal cancer cell lines

Colorectal cancer cell lines were used for motility and proliferation experiments as described in later section. These were adopted as similar immunohistochemical Two colorectal cancer cell lines (SW-480 and SW-837) were kindly sourced from Mr. Chin Ang in the Division of Surgery and Oncology, University of Liverpool. The motility and

proliferation of these cells was analysed when they were treated in vitro with recombinant GST tagged S100A8 and S100A9. There are over 50 types of colorectal cancer cell lines with a varying spectrum of genetic abnormalities, adherent growth and epithelial morphology. As with the case of pancreatic cancer cells we were interested in those with aberration of the SMAD4 gene. In addition, the stable SMAD4 positive and negative clones were SW-480 derivatives, which is why this cell line was chosen. SW837 is a rectal cancer cell line, which is SMAD4 positive in addition to this it exhibits excellent growth and motility potential. The ability to express SMAD4 protein both in the parental and stable clones was analysed using western blotting of cell lysate.

SW- 480

SW480 was established from a primary Dukes B adenocarcinoma of the colon established from a 53 years old Caucasian male (Lelbovitz et al., 1979). The cells over expresses p53 protein (Rodrigues et al., 1990) and have positive expression for expression for c-myc, K-*RAS*, H-*RAS*, N-*RAS*, *myb, sis* and *fos* oncogenes(Trainer et al., 1988).

SW-837

This cell lines was generated in the laboratory of Leibovitz, having been derived from a grade IV adenocarcinoma of the rectum. These cells are SMAD4 positive, colon antigen-3 negative and have mutation of the p53 gene (Chen et al., 1983; Nigro et al., 1989). The cells are positive for keratin by immunoperoxidase staining (Lelbovitz et al., 1979; Rodrigues et al., 1990).

Monocytic cells lines

The effects of pancreatic cancer cell conditioned media on monocytes was analysed using HL-60 cells which are a promyelocytic cell line from a 36-year-old Caucasian female with acute promyelocytic leukaemia. These cells were purchased from ATCC USA

HL-60 cells spontaneously differentiate and this can be stimulated by butyrate, hypoxanthine, phorbol myristic acid (PMA, TPA), dimethyl sulfoxide (DMSO1%), actinomycin D, and retinoic acid. The expression of S100A8 and S100A9 appears to be restricted to early stages of myeloid differentiation, therefore the human promyelocytic leukaemia cell line HL-60 has been used to study S100A8 and S100A9 expression throughout the literature (Kerkhoff et al., 1998) (Gebhardt et al., 2006) (Foell et al., 2008) (Leukert et al., 2006) (Roth et al., 2001); (Suryono et al., 2006). Untreated HL-60 cells express little or none S100A8 and S100A9 but these proteins are seen to be induced in HL-60's when the cells differentiate (Kerkhoff et al., 1998). This differentiation has been shown to be achieved in a variety of studies using a host of conditions (Rahimi et al., 2005; Shibata et al., 2004; Suryono et al., 2006).

SMAD4 expressing clones (pancreatic and colorectal)

SMAD4 negative and SMAD4 re-expressing clones of colorectal cancer cell line (SW-480) and pancreatic cancer cell line (HS-766) lines were a kind gift from Dr I Schwarte-Waldhoff (Schwarte-Waldhoff et al., 1999; Schwarte-Waldhoff and Schmiegel, 2002; Schwarte-Waldhoff et al., 2000). These were generated using a full-length coding sequence of *SMAD4* cloned into pBK-CMV expression vector using a standard calcium

phosphate co-precipitation method. Clones were transfected with either SMAD4 or an empty vector to yield both positive and negative SW-480 and HS-766 clones. Expression of SMAD4 was determined using western blotting and northern blotting analysis (Schwarte-Waldhoff et al., 1999). The SWD20, SWD14, and SWD1 cell lines are SMAD4-re-expressing clonal derivative of SW480 following stable transfection of the full-length coding sequence of *SMAD4/DPC4*. SWK3 is a control clone lacking *SMAD4*.

These cell lines were used to provide conditioned media, which was used to treat monocytic cell lines to study the effects S100A8 and S100A9 expression in them based on varying SMAD4 production. Recombinant GST tagged S100A8 and S100A9 were used to analyse the varying effect in motility and proliferation of the SMAD4 positive and negative clones.

9.2 Human tissue used in these experiments

Colorectal cancer cell lines

Two colorectal cancer cell lines (SW-480 and SW-837) were sourced from ATCC (American Type Culture Collection, USA). The motility and proliferation of these cells was analysed when they were treated in vitro with recombinant GST tagged S100A8 and S100A9.

Monocytic cells lines

The effects of conditioned media from pancreatic cancer cells on monocytes was analysed using HL-60 cell lines. These were purchased from ATCC (American Type Culture Collection, USA).

SMAD4 expressing clones (pancreatic and colorectal)

SMAD4-negative and SMAD4-positive re-expressing clones of colorectal cancer cell line (SW-480) and pancreatic cancer cell (HS-766) lines were kindly obtained from Dr I Schwarte-Waldoff (Schwarte-Waldhoff et al., 1999; Schwarte-Waldhoff and Schmiegel, 2002; Schwarte-Waldhoff et al., 2000). These SW-480 clones were utilised in motility and proliferation experiments with recombinant GST tagged S100A8 and S100A9 proteins. Both SW-480 and HS-766 clones were also utilised in conditioned media experiments assessing monocytic S100A8 and S100A9 expression.

Human Monocytes isolation from human blood

Human blood monocytes were prepared from blood by density gradient centrifugation and differential adherence technique (Lewthwaite et al., 2002). Briefly, human blood was acquired from the UK Blood Bank (Mersey). Twenty millilitres of blood was transferred into heparinised sterile universal tubes (Sterilin) to which an equal volume of phosphate buffered saline (PBS, Sigma: 137mM sodium chloride, 8.1mM sodium phosphate pH 7.4) was added. Thirty millilitres (30 mL) of this mixture of blood and PBS was then carefully layered onto 15 mL Lymphoprep™ (Axis-Shield) which is a ready-prepared, sterile and

endotoxin-tested solution for the isolation of pure leukocyte suspensions (composition: Sodium Diatrizoate 9.1% (w/v) and polysaccharide 5.7% (w/v)).

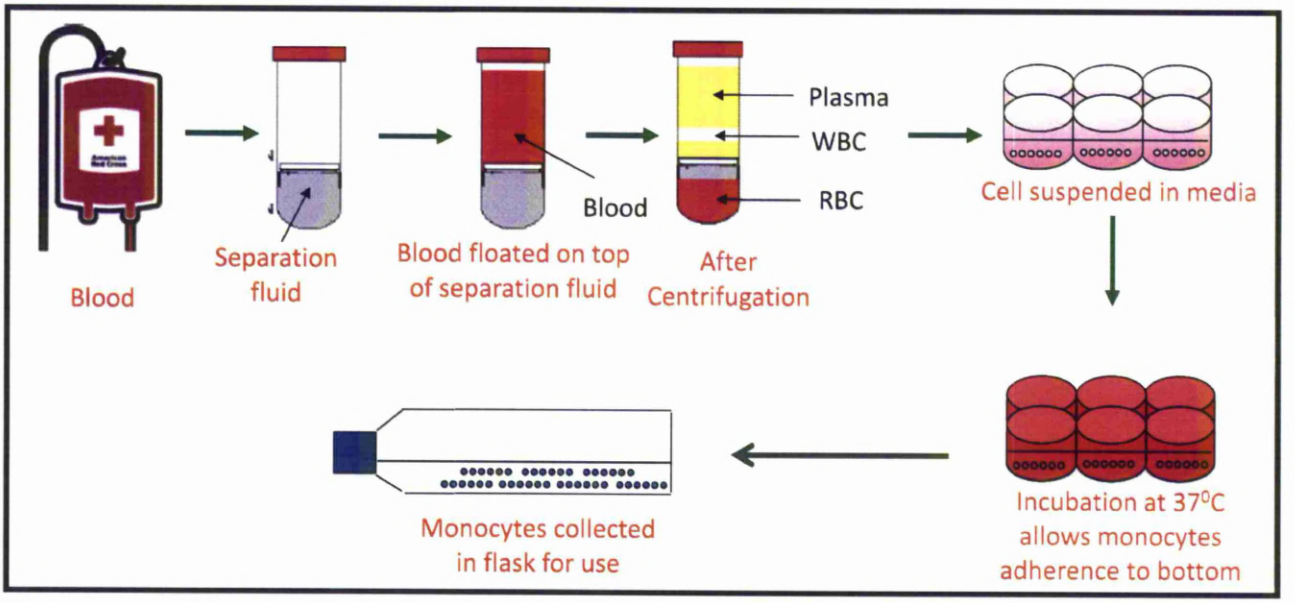


Figure 2.1: Method used to separate monocytes from blood. Three tubes are shown in order from left to right: one with lymphoprep, the second with blood floated on lymphoprep and the third after spinning and separation of different blood constituents. The WBCs separated were then suspended in media and also to incubate enable the monocytes to adhere to bottom

RPMI-1640 medium containing 2% foetal calf serum, L-glutamine 2mM (Sigma) and Penicillin-Streptomycin solution 5 mL (Sigma; composition 50U of penicillin and 50µg of streptomycin).

Monocytic density was counted using a haemocytometer and their viability was also assessed using trypan blue exclusion. Cells were then plated in 6 well plates at a density

of $2x10^6$ cells per well. Plates were incubated for 1 hour at 37°C and the media removed leaving adherent cells (monocytes) behind. Adherent cells were then washed with PBS twice and cultured in conditioned media.

9.3 Cell culture and maintenance

Maintenance of all pancreatic and colorectal cancer cell lines used was undertaken using aseptic technique in a class II laminar flow tissue culture cabinet. Cells were cultured as a monolayer at 37°C with 5% CO_2. They were routinely maintained in $75cm^2$ (T75) tissue culture flasks and were generally sub-cultured every 48 – 72 hours, when they were at a confluence of 80-90%.

The HL-60 cell line was grown in Dulbecco's Modified Eagles' Medium, supplemented with 20% Foetal Bovine Serum (Sigma), L-glutamine 2mM (Sigma) and Penicillin-Streptomycin solution 5 mL (Sigma; composition 50U of penicillin and 50μg of streptomycin). The HL-60 cell line did not form a monolayer. It remained suspended in medium at all times.

Pancreatic cancer cell lines were grown in RPMI-1640, supplemented with 10% Foetal Bovine Serum (Sigma), L-glutamine 2mM (Sigma) and Penicillin-Streptomycin solution 5 mL (Sigma; composition 50U of penicillin and 50μg of streptomycin).

The colorectal cell line SW-480 were grown in Dulbecco's Modified Eagles' Medium, supplemented with 10% Foetal Bovine Serum (Sigma), L-glutamine 2mM (Sigma) and Penicillin-Streptomycin solution 5 mL (Sigma; composition 50U of penicillin and 50μg of streptomycin). SW837 was grown in carbon dioxide free conditions using Leibovitz L-15 medium (Sigma) with all supplementations similar to the SW-480 cell lines.

SMAD4-positive and -negative colorectal (SW480) and pancreatic (HS766) cells were grown in Dulbecco's Modified Eagles' Medium, supplemented with 10% Foetal Bovine Serum (Sigma), L-glutamine 2mM (Sigma) and Penicillin-Streptomycin solution 5 mL (Sigma; composition 50U of penicillin and 50μg of streptomycin), however an additional supplementation of G418 disulphate salt solution at a concentration of 150 μg/mL was used. This maintained a selection pressure allowing only cells containing the pBK-CMV expression vector with the G418 resistance gene to grow.

All media, PBS and trypsin were warmed to 37°C prior to use. For subculturing media was removed and cells washed with sterile PBS twice to remove any residual media and cell debris. A ready-made Trypsin-EDTA solution from Sigma (composition: 0.5g porcine trypsin and 0.2 g EDTA in Hanks' Balanced Salt Solution with phenol red) was layered to cover the cells (2 mL per T75 flask used). Cells were incubated with this media for a few minutes until no longer adherent to the flask base. Microscopic confirmation of this non-adherence was undertaken by light microscopy. The subculture ratio and trypsin time for each cell line used is shown in the table 2.7.

CELL LINE	SPLIT RATIO	TRYPSIN TIME
HL-60	1:8	Not Required
BxPC3-3	1:3	5 minutes
PANC-1	1:5	3 minutes
CFPAC-1	1:4	3 minutes
SUIT-2	1:10	3 minutes
MIA PaCa-2	1:8	2 minutes
SW480 (wild type & clones)	1:6	3 minutes
HS766 (clones)	1:4	3 minutes

Table 2.7: Table showing the sub-culture ratio and trypsin time for various cell lines

Once the cells were free from the base of the flask the trypsin was neutralised with 10 ml of the serum containing medium. Cells were gently titrated to produce a single cell suspension and then re-plated at a density appropriate for each cell line (Table 2.7).

HL-60 cells are non-adherent and therefore once extracted from the T75 flask were centrifuged at 100 RCF for 8 minutes following which they were washed in PBS and centrifuged as before. The cell pellet was then suspended in 10 mL of DMEM and then spilt in a ratio of 1:8.

Freezing and thawing cells

Cells were harvested with trypsin as outlined in the above. Cells from one T75 flask were then placed in a test tube and centrifuged at 100 RCF for 8 minutes. Cells were then washed in PBS and centrifuged again as before. The supernatant (PBS) was then removed, the cells were resuspended in 1mL of freeze media (65% serum free media [RPMI/ DMEM/L-15], 25% Foetal Bovine Serum, 10% DMSO) and placed in a cryovial.

The cryovial was initially incubated on ice for about 1 hour and then placed at -80°C for 24 hours prior to storage in liquid nitrogen. When required, cells were defrosted at 37°C and immediately transferred into pre-warmed PBS, washed twice and centrifuged at 100 RCF for 8 minutes before being suspended in the appropriate culture media at a density of $3x10^6$ cells per T75 flask. HL-60 cancer cell lines were frozen in exactly the same way as described in the above paragraph, except the freezing media was different (95% HL-60 culture media and 5% DMSO).

9.4 Cell culturing in conditioned media

HL-60 cells and primary human monocytes were cultured, for defined time periods in conditioned media derived from pancreatic cancer cell lines (PANC-1, SUIT-2, BxPC3, and CFPAC-1) and SMAD4 re-expressing colorectal (SW-480) and pancreatic cancer (HS-766) clones.

Collection of conditioned media from pancreatic cancer cell lines

Pancreatic cancer cell lines were plated in a T75 flask at the density previously described. After 48 hours when cells were approximately 85% confluent, spent medium was collected and centrifuged at 300 g for 8 minutes to remove cell debris. Then the media was filter sterilised using a 0.2 μm filter and warmed to 37°C. Fresh media was then added to the conditioned media in a ratio of 1:3 such that there was 1 part fresh media and 3 parts conditioned media. Conditioned media was also collected for control purposes from HL-60 cells or primary human monocytes or media derived from MEF (mouse embryo fibroblast) cell lines (obtained from Dr Boyd's labs in the Division of Surgery and Oncology). Conditioned media from SMAD4-positive and negative colorectal (SW480 derivatives) and pancreatic (HS766 derivatives) cancer cell lines were obtained as mentioned above and treated in exactly the same manner.

Culturing of HL-60 cells and human monocytes in conditioned media

HL-60 or primary human monocytes were plated in six well plates at a density of $2x10^5$ cells per well. Two and half millilitres of conditioned media or cytokine-supplemented media was then added to each well. Monocytes and HL-60 cells were incubated for 24- and 48-hour period following which the cells were extracted from each well and pooled. The cells were then spun at 100 RCF for 8 minutes followed by a wash in PBS. The cells were then lysed using RIPA buffer for protein analysis using western blotting experiments.

9.5 Protein analysis of conditioned media

Conditioned medium from pancreatic cancer cell lines and HL-60 cancer cell lines was analysed for the presence of secreted cytokines (TGF-β1 and VEGF-A in PDAC cell lines) and secreted forms of S100A8 and S100A9 (in HL-60 cell lines). Cells (PANC-1, BxPC3-3, CFPAC-1, SUIT-2 and HL-60) were plated at low density in T75 flasks using serum free media. The viability of the cells prior to collection of medium was assessed using trypan blue exclusion and medium was collected as described previously. The media was then centrifuged at 100 RCF for 8 minutes to remove cell debris the supernatant (conditioned media) then removed. The medium was then filter sterilized with a 0.2 μm filter and then aliquoted into 1 mL tubes. It was vacuum centrifuged at 30°C for 3-4 hours until a 10 fold reduction in volume occurred. Aliquots were then pooled together and a protein assay preformed as described previously.

9.6 Western Blotting

Cell Lysate preparation

Cell pellets of monocytic cells (HL-60 and human monocytes) treated with conditioned media, pancreatic cancer cell lines (PANC-1, SUIT-2, BxPC3, and CFPAC-1) and SMAD4 re-expressing colorectal (SW-480) and pancreatic (HS-766) cancer cell lines were lysed on ice with RIPA buffer (50 mM Tris, 150mM Sodium Chloride, 0.5% Sodium Deoxycholate, 1% Igepal [a nonionic, non-denaturing detergent], 0.1% Sodium Dodecosulphate, made up to pH of 8.0). A protease inhibitor cocktail tablet (Roche) was

added to a 10mL tube of RIPA buffer prior to use. Five hundred microlitres of RIPA buffer was added to each pellet and cells suspended in the solution and incubated on ice for 30 minutes. Cells were then disrupted using hydrodynamic shearing with a 21 gauge needle and incubated for a further 30 minutes. Samples were then spun in a centrifuge at 10,000 RCF at 4°C for 10 minutes to remove any cellular debris, and the consequent supernatant was collected and utilised.

Bicinchoninic acid (BCA) Protein assay

A dilution curve of standard protein concentrations was created using bovine serum albumin (Sigma) made up in RIPA buffer using a BCA Protein Assay kit (Pierce). The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein.

Preparation of Diluted Albumin (BSA) Standards

To prepare diluted albumin standards, the content of one albumin standard ampoule at 2000 µg/mL (provided in the assay kit) was diluted serially using RIPA cell lysis buffer to produce a set of diluted standards as shown in Table 2.8. The BCA™ Working Reagent (WR) was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B (50:1, Reagent A: B) which were provided in the kit. Fifty microlitres of each sample and standards A-I were added in duplicate to each cuvette. This was followed by the addition of 1 mL of the working reagent to each sample and standard. This mixture was then covered and incubated for 2 hours at room temperature. The standards were read on the

spectrophotometer set to 562 ηm using the BCA function. Following standardisation, the samples were then read to determine the protein concentration of each sample.

Vial	Volume of Diluents	Volume of BSA	Final BSA concentration
A	0 µL	300 µL of stock	2000 µg/mL
B	125 µL	375 µL of stock	1500 µg/mL
C	325 µL	325 µL of stock	1000 µg/mL
D	175 µL	175 µL of Vial B	750 µg/mL
E	325 µL	325 µL of Vial C	500 µg/mL
F	325 µL	325 µL of Vial E	250 µg/mL
G	325 µL	325 µL of Vial F	125 µg/mL
H	400 µL	100 µL of Vial G	25 µg/mL
I	400 µL	0 µL	0 µg/mL

Table 2.8: Table showing dilution of Albumin Standards used to make up Standards for BSA protein assay.

Denaturing of protein

Protein samples were diluted to a specified concentration using RIPA buffer (Lysis Buffer) and 5X SDS sample buffer (10% SDS, 50% glycerol, 600 mmol TRIS adjusted to pH 6.8, 0.05% Bromophenol blue made up in water) to give final volumes of 100µL (with a final concentration of 1X SDS sample buffer). This mixture was then heated at 100°C for 15 minutes.

Separation of protein by SDS-Page gel electrophoresis

The detection of S100A8 and S100A9 proteins was undertaken using 15% Tris-tricine (4.5 mL of 30% Acrylamide mix, 1.4 mL water, 2.9 mL Gel buffer [Tris 300 mM, 1mM sodium dodecyl sulphate [SDS], made up to 100 mL at pH of 8.4], 1 mL Glycerol, 75µl of 10% Ammonium persulfate [APS], 7 µL TEMED) based SDS polyacrylamide gel electrophoresis (SDS-PAGE).

The detection of SMAD4, TGF-β1 and VEGF-A was undertaken on a 12% tris-glycine based gels (4mL water, 3.3mL of 30% Acrylamide mix, 2.5 mL of 1.5M TRIS pH 8.8, 0.1 mL of 10% SDS, 0.1mL of APS, 10µL TEMED).

Larger proteins were analysed on lower percentage gels; therefore, for detection of SMAD4 a 10% polyacrylamide gel was used in comparison to a 15% polyacrylamide gel which was used for detection of S100A8 and S100A9. Gel glass plates (0.75 mm) were cleaned with 70% ethanol, air-dried and mounted on a Bio-Rad kit.

An SDS Polyacrylamide separating gel was poured between the plates to within 1.5cm of the top, covered with distilled water, left to set for 20 minutes following which the water was removed with blotting paper, and a stacking gel (For Tris-tricine gels: 0.82 mL 30% Acrylamide mix, 2.1 mL Gel buffer, 3.4mL Water 40 µL of 10% APS, 5µL TEMED; For Tris-glycine gels: 2.7 mL water, 0.67 mL 30% Acrylamide mix, 0.5mL 1M TRIS pH 6.8, 40µL 10% SDS, 40µL 10% APS 4µL TEMED) was added. A 10 well comb was placed into the stacking gel and allowed to set for 15 minutes before removal of the comb and washing out of the wells with water. The gel and plates were then transferred to a

Mini Protean III electrophoresis chamber (Bio-Rad). In the case of Tris-tricine gels, a separate anode (TRIS 1M pH 8.9) and cathode buffer (100mM TRIS, 100mM Tricine, 3.5mM SDS) were added to the outer and central chamber respectively, whereas in Tris-Glycine gels the same electrophoresis buffer (30mM TRIS, 192mM Glycine, 1.3 mM SDS) was added to both chambers. The sample was loaded alongside similar volumes of Seeblue marker which is a pre-stained molecular weight marker (Invitrogen, technology, USA). Gels were run initially at 80-90 volts for 1 to 1.5 hours until adequate separation of marker was observed.

The gel apparatus was then dismantled, the stacking gel removed and transferred onto a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The gel and membrane were sandwiched between 3mm Whatman chromatography paper (Sigma U.K) and sponges. This assembled apparatus was constructed in transfer buffer to prevent any air bubbles entering between the gel and membrane. The transfer apparatus was then returned to the chamber, along with an ice block for cooling, the chamber filled with the buffer (200mL Methanol, 30mM TRIS, 192mM, Glycine, 1.3 mM SDS) and transferred for 1 hour at 110V with a magnetic stirrer allowing circulation of transfer buffer.

Blocking

Once removed from the transfer chamber the nitrocellulose membrane was rinsed in PBS Tween (1L PBS solution with 1mL Tween) and blocked (5% blotting grade non-fat dry milk powder, 2% Bovine serum albumin and PBS-tween) overnight at 4°C when probing

for S100A8 and S100A9 and for 1 hour at room temperature when probing for SMAD4, TGF β-1, and VEGF-A blocking.

Immunoblotting

Incubation with primary antibodies

Following blocking, membranes were agitated at room temperature with primary antibodies, which were diluted with 3% milk powder in PBS Tween at concentrations as outlined below, based on suppliers recommendations. Primary antibodies applied were either monoclonal mouse anti-S100A8 (Santa Cruz biotechnology, diluted 1:100) or polyclonal rabbit anti-S100A9 (Santa Cruz biotechnology, diluted 1:100) or monoclonal mouse anti-β-Actin (Santa Cruz biotechnology, diluted 1:10000), or polyclonal mouse anti-TGF-β1 (Abcam, UK, diluted 1: 1000) or polyclonal mouse anti-SMAD4 (Santa Cruz, biotechnology, diluted 1:2000). Following primary incubation for 1 hour at room temperature, the membranes were washed for 4 cycles each lasting 15 minutes in PBS Tween at 37^0C. This was then followed by incubation with secondary antibodies, for 1 hour each, at room temperature, with continuing agitation. The blot was washed in warm PBS-Tween for four cycles each for 15 minutes.

Incubation with Secondary antibodies

Based on the primary antibody utilized, the secondary antibodies were either anti–mouse IgG, Horse Radish Peroxidase linked whole antibody (HRP), (DAKO 1mg/mL diluted 1:2000) or anti-rabbit IgG, HRP linked whole antibody, (DAKO 1mg/mL diluted 1:1000). The secondary antibody was added to a solution containing 3% milk in PBS-

Tween. Membranes were incubated with secondary antibodies for 1 hour at room temperature with continuous agitation and then washed with PBS Tween for 4 cycles each lasting 15 minutes.

Developing Western Blots

Protein bands on membranes were visualised by exposing them to medical x-ray film. For this, the membranes were first incubated in Enhanced Chemo-Luminescence (ECL) reagent plus for 5 minutes. Equal quantities of the Enhanced Luminal and Oxidising reagents were combined and applied to cover the entire membrane. The membrane was then blotted dry, wrapped in cling film and secured in a Kodak light safe developing cassette. The membrane was then exposed to medical x-ray film at a range of exposure lengths to optimise the imaging of the various proteins being studied. The film was consequently developed in Kodak developer and fixer for two minutes respectively and then rinsed in tap water and allowed to air dry.

Membrane stripping for β-actin probing

The membranes were then probed with β-actin, which enabled examination and documentation of equal protein loading for each lane of the gel. The membranes, which had ECL reagent on them, were first washed 2 x 15 minutes with PBS-Tween and then incubated with stripping buffer (Sigma U.K) at room temperature for 20 minutes. This was followed by two cycles of PBS-Tween washes before mouse monoclonal anti-β-actin antibody (dilution 1:10,000) was added to the membrane and incubated either overnight at 4^0C or for 1 hour at room temperature. The subsequent steps were similar to standard

western blotting with washing of primary antibody, application of secondary and developing as described previously.

9.7 Effects of conditioned pancreatic cancer cell line media on expression of S100A8 and S100A9 in human monocytes and HL-60 cell lines

Data from immunohistochemical staining of the pancreatic cancer TMAs revealed a significant association between the SMAD4 status of the tumours and the number of S100A8-positive cells (Sheikh et al., 2007). SMAD4 negative tumours significantly correlated to the number of S100A8-postive cells in the tumour stroma ($p = 0.0023$; Fisher's Exact Test). This was not the case for S100A9-positive cells which were independent of the SMAD4 status of the tumours ($p = 0.21$; Fisher's Exact Test). Moreover, when the ratio of S100A9 to S100A8 cells in SMAD4-positive and SMAD4-negative tumours was examined, a striking difference between the two groups was noticed. A median of 1.67 fold (IQR 1.21 to 2.4) greater S100A9 to S100A8 positive cells in SMAD4-expressing tumours was observed compared with a median of 3.16 (IQR 1.89 to 6.54) fold greater S100A9 to S100A8 positive cells in SMAD4-negative tumours ($p<0.004$, Mann-Whitney U test). Thus, a strongly negative relationship between the expression of SMAD4 in tumours cells and the expression of S100A8 in stromal inflammatory cells was established. In addition to these finding as described in the previous section, I also was able to establish a correlation between pancreatic cancer nuclear TGF-β1 expression and the number of S100A8- and S100A9- positive cells in the stroma indicating a link between cancer cells and stromal expression of these S100 proteins.

Effects of conditioned media on human monocytes

In order to further elucidate the association pancreatic cancer has with the expression of S100A8 and S100A9 in stromal monocytes, incubation of human monocytes (n=3) with conditioned media, derived from 4 pancreatic cancer cell lines (PANC-1, SUIT-2, CFPAC-1 and BXPC3-3) was undertaken. Incubation of monocytes with conditioned medium from pancreatic cancer cells lines was associated with an increase in S100A8 and S100A9 expression after 48 hours of incubation compared to the controls. The level of expression of both proteins varied based on the cell line that the condition medium was derived from. Additionally, an increase in one S100 protein expression was not always mirrored by a similar level of increase in the other S100 protein with medium derived from the same cell line as in the case of medium derived from SUIT-2 cell lines (Figure 2.10).

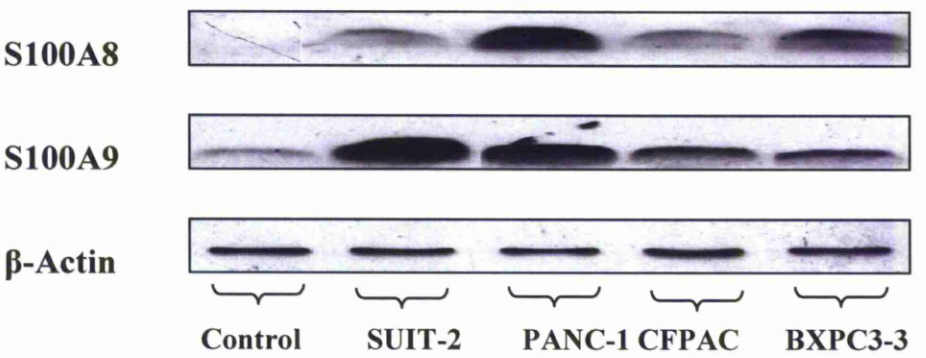


Figure 2.10: Western blots demonstrating S100A8 and S100A9 expression in human monocytes when treated with media from different pancreatic cancer cell lines and controls.

Effects of conditioned media on HL-60 cell line

The effects of conditioned media from pancreatic cells lines on the expression of S100A8 and S100A9 in the HL-60 monocytic cell line was also evaluated (n=5). Media derived from all 4 pancreatic cancer cell lines (PANC-1, SUIT-2, CFPAC-1 and BXPC3-3) produced an increase in S100A8 and S100A9 expression at 48 hours compared to controls (Figure 2.11). The level of increase in expression varied depending on which cell line the media was derived from. The two controls used were spent media derived from HL-60 cell lines themselves (control) and Mouse embryonic fibroblasts (MEF) cell line, which is an inert non-cancer cell line.

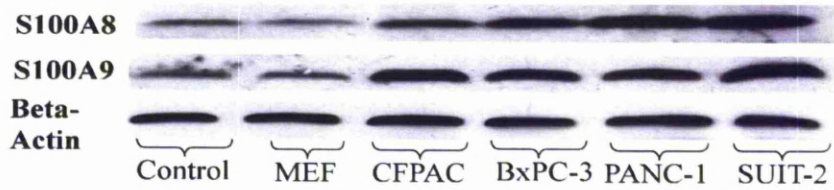


Figure 2.11: Western blots demonstrating S100A8 and S100A9 expression in HL-60 cells at 48 hours after treatment with media from pancreatic cancer cell line and controls (control: media from HL-60 cell lines and MEF: media from mouse embryonic fibroblasts)

SMAD4 status of cell lines used

Our findings on IHC analysis indicated an association between S100A8 and S100A9 expressing cells and tumour SMAD4 status. In addition conditioned media from pancreatic cancer cells induced expression of these 2 proteins in human monocytes and cancer cell lines alike. Therefore, I proceeded to evaluate the presence of SMAD4 in the pancreatic cancer cell lines used in the above experiments. According to the literature PANC-1 and SUIT-2 cell lines were SMAD4 positive whereas CFPAC-1 and BxPC-3 were SMAD4 negative. Independent verification by western analysis of cell lysate revealed loss of SMAD4 in CFPAC-1 and BxPC-3 but also in SUIT-2 cell line with PANC-1 being positive for SMAD4 (Figure 2.12). The loss of SMAD4 in the SUIT-2 cell line may be a result of acquired mutation to the *SMAD4* gene acquired as a result of multiple cell passages.

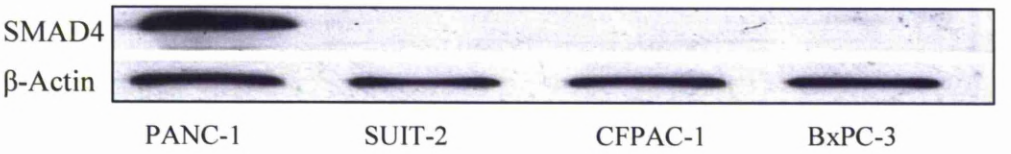


Figure 2.12: Western blots for demonstrating SMAD4 expression in four pancreatic cancer cell line lysate with β-Actin as control

Using conditioned media from various pancreatic cancer cell lines which were both SMAD4 positive (PANC-1) and negative (BxPC-3, CFPAC-1 and SUIT-2) did not reveal a differential induction in the expression of S100A8 and S100A9 in monocytes and HL-60 cell lines based on the SMAD4 status of the cancer cell from which the condition media was harvested. Therefore, media derived from stable SMAD4 positive and

negative colorectal and pancreatic clones was used to further define if differential expression of S100A8 and S100A9 occurred when conditioned media was derived from the same parental cell line which has both SMAD4 positive- and -negative clones.

Effects of conditioned media derived from stable SMAD4 positive and negative colorectal and pancreatic cancer cell lines

Media derived from stable (SMAD4 positive and negative clones) colorectal and pancreatic cancer cell lines SW480 (n=6) and HS766 (n=3) respectively was used to treat HL-60 cell lines in the same experimental conditions as described previously. Induction or suppression of S100A8 and S100A9 proteins in HL-60 cells was analysed using western blots and densomertic assessment of bands.

The experiments were performed using conditioned media from SW480 clones SWD20, SWD14 and SWD1 (SMAD4-positive clones) and SWK3 (SMAD4-negative clone). These cell lines have varied expression of SMAD4 which is demonstrated by Western blot analysis (Figure 2.13). SWD1 and SWD14 expresses less SMAD4 compared to SWD20 while in SWK3 a faint band was observed.

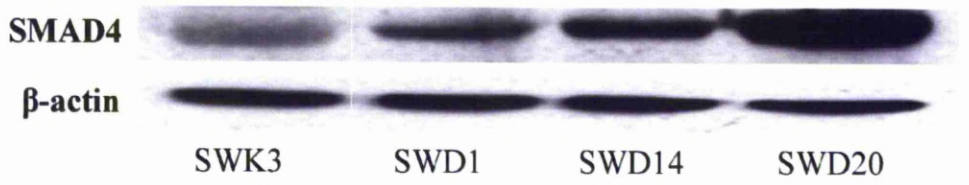
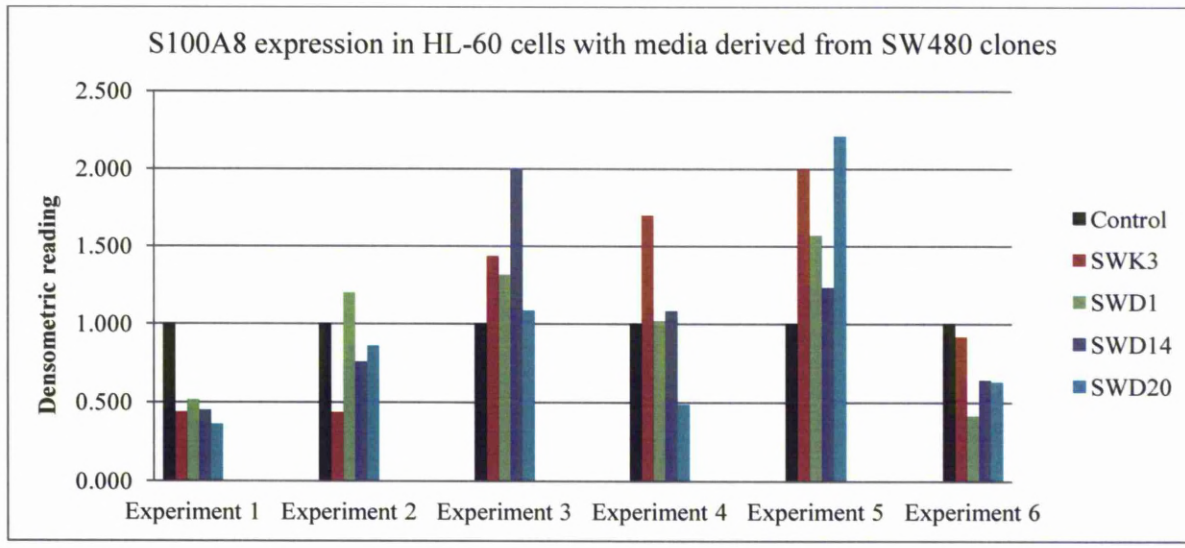


Figure 2.13: Figure showing western blot data from cell lysate of SW480 clones (SMAD4 -positive clones and -negative clones) after a delayed 15 minutes of exposure.

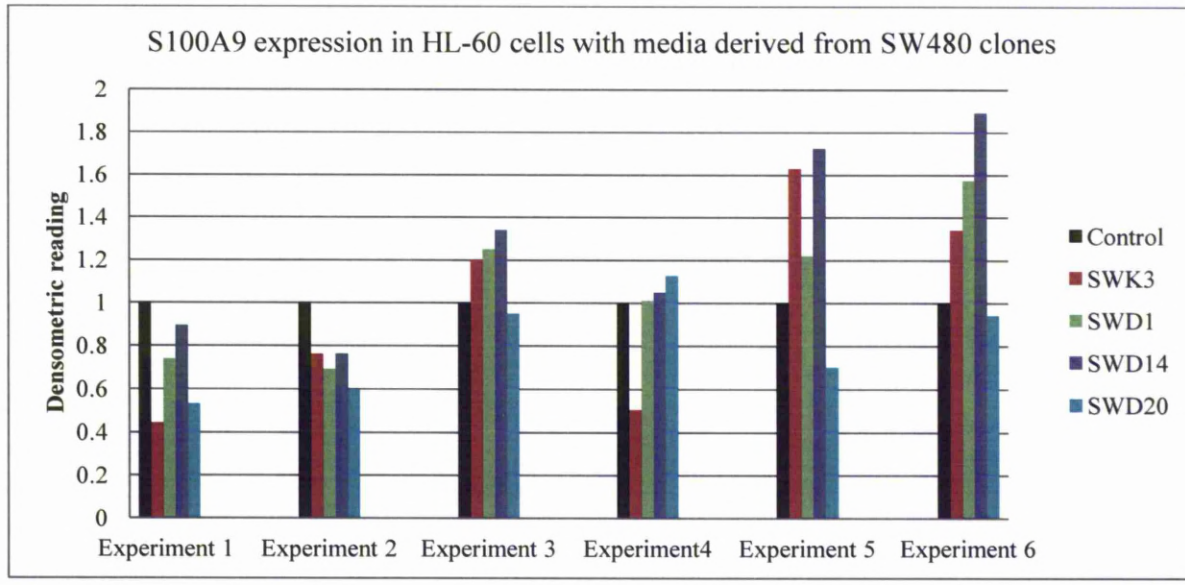
Densometric readings of S100A8 and S100A9 protein bands induced in HL-60 cells following treatment with conditioned media from the SMAD4-postive and –negative clones for each experiment are shown in the graphs below. These indicate an experimental variably in the expression of S100A8 and S100A9 inspite of all experimental conditions being consistent. The control displayed as a black bar in each of the figures (Figure 2.14 and 2.15) was the standard control used for these experiments as before which is spent media derived from HL-60 cells. Densometric readings for each band density plotted has been normalised to control i.e. the densometric reading of all protein bands has been divided by the densometric reading of the control. It can be seen that compared to the controls no consistent result can be deduced as in some experiments conditioned media caused an induction of S100A8 and S100A9 proteins, with other experiments showing reduced expression. Owing to lack of consistency in the experiments, no safe conclusion could be made. Additionally, and more importantly no variance in the levels of S100A8 and S100A9 expression was seen between media derived from SMAD4 re-expressing cells compared to the negative cell lines.

Similarly, a varied response in terms of the expression of S100A8 and S100A9 induced by the media derived from HS766 clones was also observed (Figure 2.15). The two

graphs below show densometric readings from the experiments performed using media form HS766 clones where HSD4 and HSD8 are SMAD4 positive clones and HSK3 and HSK6 are SMAD4 negative clones. The readings have been normalised to actin in a similar manner to SW-480 cell line experiments. It can be seen that media from clones tended to increase the expression of S100A9 in the HL-60 cells, with S100A8 expression being suppressed compared to controls. These results did not achieve significance and were not consistent to make any conclusions about variation in expression of S100 protein from media sourced from SMAD4 re-expressing cells compared to the SMAD4-negative cells.

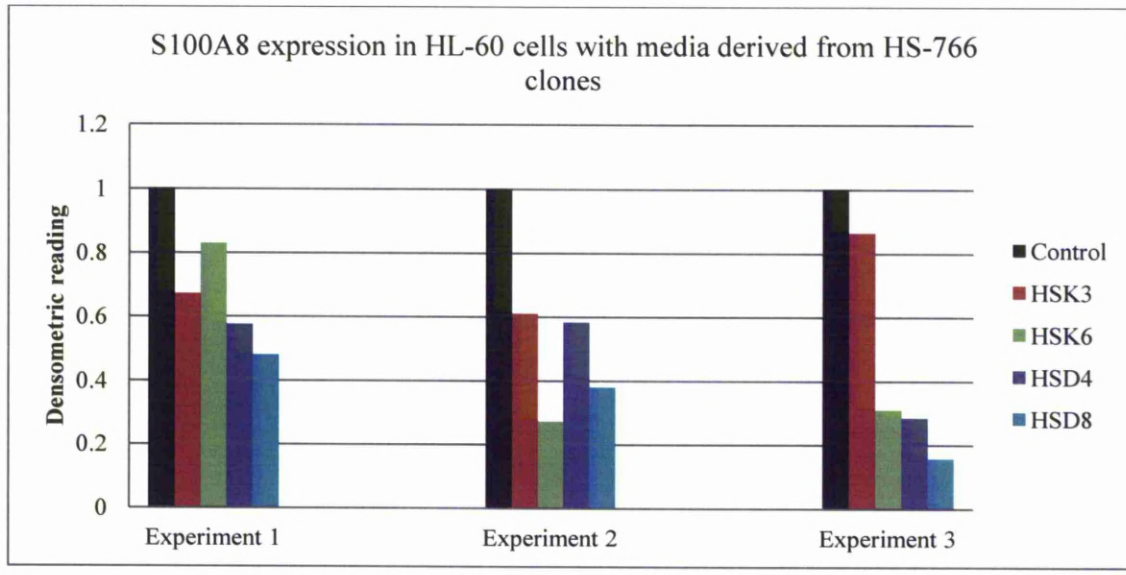


A

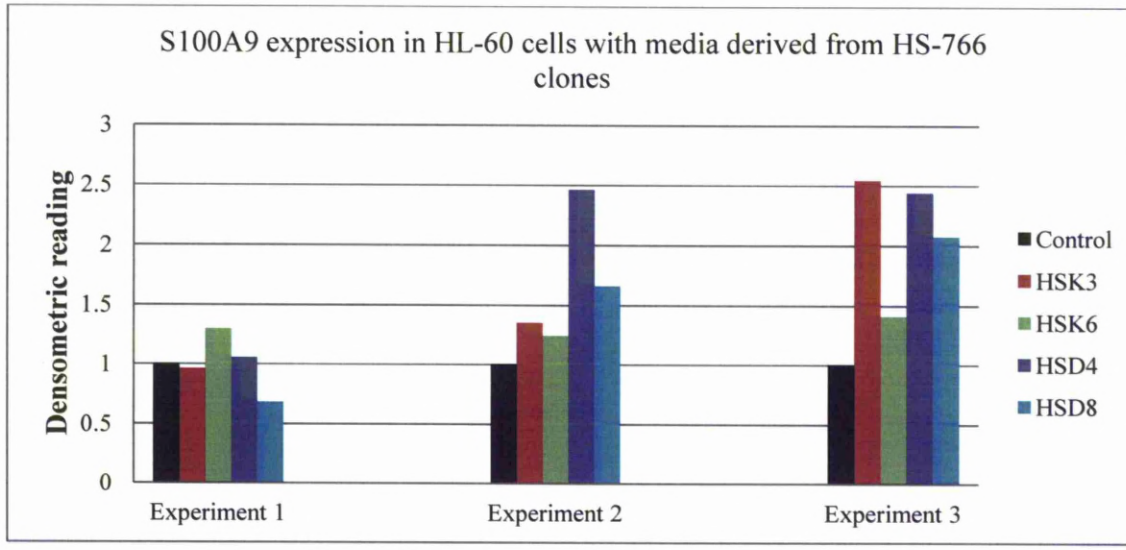


B

Figure 2.14: S100A8 (A) and S100A9 (B) expression in HL-60 cell lines following treatment with conditioned media derived from SMAD4 positive and negative clones of SW480. Densometric readings from western blots have been normalised to actin and control (media derived from HL-60 cell lines).



A



B

Figure 2.15: S100A8 (A) and S100A9(B) expression in HL-60 cell lines following treatment with conditioned media derived from SMAD4 positive and negative clones of HS766. Densometric readings from western blots have been normalised to control (media derived from HL-60 cell lines).

9.8. The expression of S100A8 and S100A9 and its association with TGF-β1 and VEGF-A

In a mouse model experiment VEGF-A and TGF-β1 produced by primary tumours were implicated as drivers of the pre-metastatic niche producing expression of S100A8 and S100A9 in tissue (Hiratsuka et al., 2006). I was able to demonstrate the presence of VEGF-A and TGF-β1 in pancreatic cancer cell lines and as secreted proteins in conditioned serum free media from these cells (Figure 2.16). The blots demonstrate the presence of TGF-β1 and VEGF-A to be present both in the cells and also as a secreted factor in conditioned media. The absence of β-actin bands in the serum free conditioned media indicate that both these proteins are secreted and not a result of cell lysis causing contamination. It is interesting to note here that in the cancer cell lysate multiple bands of VEGF-A are seen which represent isomers of the protein and only a single band is detected in the medium (secreted form)

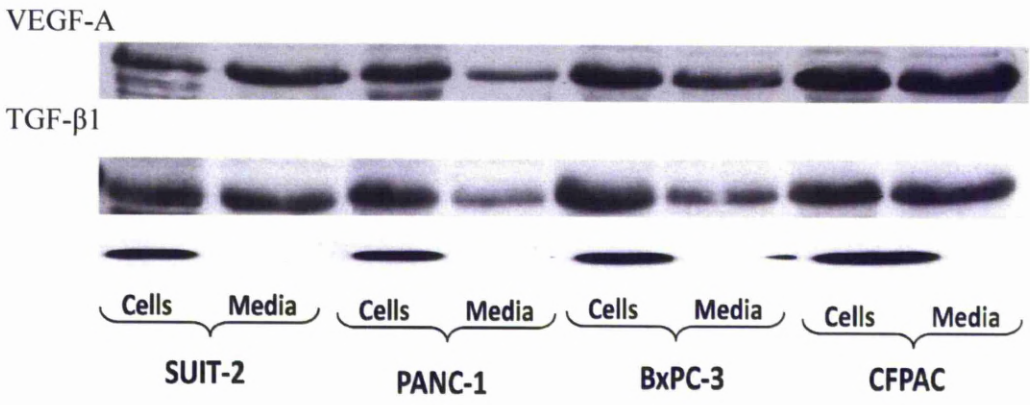


Figure 2.16: Western blots indicating the presence of TGF β1 and VEGF-A in cell lysate and conditioned media concentrate derived from four pancreatic cancer cell lines (equal protein loading 10μg/ml for both cell lysate and media in each lane).

9.9 Discussion

The immunohistochemical staining and analysis of the pancreatic cancer TMA demonstrated high levels of S100A8- and S100A9- expressing monocytes in the dense pancreatic stroma. Moreover, the expression of these proteins was linked to the SMAD4 status of the tumour cells. These proteins have been studied in various setting primarily in cells of myeloid origin (Roth et al., 2001) and their expression profile has been detailed by Zwadlo *et al.* (Zwadlo et al., 1988). The authors demonstrated the presence of both S100 proteins in immature monocytes which were expressed specifically, at early stages of monocyte differentiation showing a gradual loss during maturation to macrophages (Zwadlo et al., 1988) with the acquisition of other surface markers (Nacken et al., 2003). They initially express both S100A8 and S100A9 and as they mature S100A8 expression ceases leaving only S100A9 which is also subsequently lost as the cell matures further (Zwadlo et al., 1988).

Based on the immunohistochemical analysis it was observed that stroma from SMAD4-negative tumours had significantly fewer numbers of S100A8 positive cells compared to SMAD4-positive tumours (Sheikh et al., 2007). Moreover, when the ratio of S100A9 to S100A8 cells in SMAD4-positive and SMAD4-negative tumour stroma was examined, a difference between the two groups was noticed. A median of 1.67 fold greater S100A9 to S100A8 positive cells in SMAD4-expressing tumours was observed compared with a median of 3.16 fold greater S100A9 to S100A8 positive cells in SMAD4-negative tumours. It therefore seemed possible that the phenotypical profile of the monocytes, that is their level of maturity was related to the SMAD4 status of the tumour (Sheikh et al.,

2007). This led to the potential hypotheses that the status of the tumour seemed to have a bearing on the expression pattern of S100A8 and S100A9, which could be further interpreted as the tumour influencing the maturation and expression of these proteins. Referring back to the Hiratsuka *et al.* paper (Hiratsuka et al., 2006) S100A8 and S100A9 expression was induced in myeloid and endothelial cells in the lung tissue of mice by secreted soluble factors such as TGF-β, TGF-α and VEGF-A. These soluble factors were derived from the distant primary cancer cells. This presence of S100A8 and S100A9 in the lung tissue primed the microenvironment in what has been termed the premetastic phase affecting motility, aiding migration and implantation of the tumour cells. We postulated that the same process or a similar process could be occurring in the pancreatic cancer microenvironment. More recent literature not published at the time when I was undertaking these experiments has shown that systematic inhibition of S100A8 and S100A9 proteins with antibodies inhibited metastatic disease (Yan et al., 2010) and S100A9-deficient mice show reduced accumulation of MDSC cells in premetastatic sites further substantiating the role for S100A8/A9 in formation of premetastatic niches. Similarly, the presence of elevated levels of S100A8/A9 in sera of tumour-bearing wild-type mice, prior to any evidence of metastasis suggests that these proteins amplify pro-tumour responses, eventually leading to malignancy (Ichikawa et al., 2011).

I therefore undertook experimentation assessing firstly the influence conditioned media from pancreatic cancer had on the expression of S100A8 and S100A9 in monocytic cell lines and secondly whether the SMAD4 status of the cancer cells affected this expression. The results indicated an increase in expression of S100A8 and S100A9 in both HL-60

cell lines and human primary monocytes alike. The levels of expression induced in monocytes by the conditioned media from pancreatic cancer cell lines varied from cell line to cell line, which is in keeping with the differential genotype and protein expression they demonstrate. Moreover, negative SMAD4 state (in SUIT-2, CFPAC-1, and BxPC-3 cell lines) did not show a differential ability to induce the expression of S100A8 and S100A9 proteins in comparison to the SMAD4 positive cell line (PANC-1).

The treatment of monocytes and monocytic cell lines with conditioned media from pancreatic cell lines failed to demonstrate differential expression of S100A8 and S100A9, based on the SMAD4 status of the tumour; I therefore undertook further experimentation with conditioned media from SMAD4 re-expressing colorectal and pancreatic cancer cell lines. These cell lines in other experiments have shown to have a differentially expressing protein profile based on their SMAD4 status (Volmer et al., 2004) (Stuhler et al., 2006; Volmer et al., 2005). In experiments utilizing human pancreatic cell lines, restoration of SMAD4 in cell lines demonstrates that SMAD4 can control the angiogenic switch (Schwarte-Waldhoff et al., 2000). Analysing the secretomes and cell lysate of SW-480 colonic cancer cell lines using two dimensional gel electrophoresis, demonstrated the expression of over 47 proteins, which had a SMAD4 dependent expression pattern (Stuhler et al., 2006). Conditioned media from both these pancreatic (HS-766) and colorectal (SW-480) cancer cell lines failed to produce a differential expression of S100A8 and S100A9 based on SMAD4 status of the tumour. This could be a result of clonal variation or the fact that SMAD4 expression in tumour cells did not influence the expression of S100A8 and S100A9 in monocytes.

My results indicated that conditioned media from pancreatic cancer cell lines induce the expression of S100A8 and S100A9 in monocytes which was a result of mediators produced by the cancer cells and secreted into the media in which they grow. To evaluate this I analysed the conditioned media of pancreatic cancer cells lines for the presence of two important mediators TGF-β1 and VEGF-A. These mediators have well established pathways in monocytic and macrophage cellular response inducing a variety of proteins in them (Coffelt et al., 2009; Hiratsuka et al., 2006; Siveen and Kuttan, 2009). VEGF-A is a main driver for angiogenesis in pancreatic cancer (Korc, 2003). Pancreatic cancer angiogenesis is not controlled by the expression of VEGF-A alone but is a complex balance of other signalling molecules such as metalloproteinases and platelet derived growth factors (PDGF) (Korc, 2003; Song et al., 2005) which are present in the microenvironment. Similarly, TGF-β1 has a multifaceted relationship in the pancreatic cancer having growth mediator and tumour suppressive effects (Truty and Urrutia, 2007b). The pancreatic cancer cells in my studies exhibited levels of both of these mediators in the cell lysate and as secreted molecules into the medium alike, which are in keeping with what has been established in the literature (Bellone et al., 2006). Cytokine profiling studies indicated a host of different mediators produced by pancreatic cancer cells however, no study has been published detailing the cytokine profile of pancreatic cancer cells based on their SMAD4 status. The presence of TGF-β1 and VEGF-A in pancreatic cancer cell lysate and in conditioned media suggested their possible role in inducing S100A8 and S100A9 in monocytes. Further evaluation by members of Dr. Costello's group have demonstrated the induction of both S100 proteins when monocytes

expressing these proteins were treated with VEGF-A an TGF-β1. Nedjadi *et al.* has presented data (not published) using cytokine antibody arrays, demonstrating the presence of numerous cytokines including TGF-β1 and VEGF-A in pancreatic cancer cells lines, which seems to be induced when pancreatic cancer cells are treated with recombinant S100A8-GST and S100A9-GST proteins. This would suggest a channel of crosstalk with the S100 proteins affecting tumour cytokine profile.

Results from my experiments provided a further insight into the relationship between S100A8 and S100A9 proteins and cancer cells providing further evidence to the data from the TMA analysis (Sheikh et al., 2007). Even though using conditioned media from pancreatic cancer cells induced expression of S100A8 and S100A9 in monocytes, the SMAD4 status of the cancer cells did not shown a differential expression pattern as determined by analysis of the TMA. In order to further unravel this relationship, recombinant GST tagged S100A8 and S100A9 were generated and used to evaluate the effects on motility and proliferation of cancer cells.

CHAPTER 10

PRODUCTION OF RECOMBINANT GLUTATHIONE S-TRANSFERASE (GST) TAGGED S100A8 AND S100A9

10.1 Preparation and Transformation of competent *Escherichia coli*

The Glutathione S-transferase (GST) gene fusion system was used for production and purification of fusion proteins produced in *E. Coli.* Plasmids encoding S100A8-GST and S100A9-GST fusion proteins in the pGEX4T-1 vector were a kind gift from Professor Y. Maru, at the Tokyo Women's Medical University School of Medicine, Japan (Hiratsuka et al., 2006).

Competent *E. coli* BL21 and *E. coli* XL1blue were used for generation of S100A8-GST and S100A9-GST respectively. The coding sequence of S100A8 was cloned into the pGEX4T-1 vector by EcoRI, whereas S100A9 was cloned by directional cloning using a BamHI and Xho restriction site.

Competent *E. coli* XL1blue cells, acquired from Stratagene, were removed from storage at -80°C, and thawed on ice aliquotting 100 µl to which 1.7µl of β-mercaptoethanol was added. Following incubation on ice for 5 minutes, 5µl of plasmid reconstituted in water was added and left to incubate for a further 30 minutes. This mixture was then incubated for 45 seconds at 42°C and resuspended in 1mL Luria-Bertani (LB) media (10 g Bacto tryptone, 5g Bacterial yeast extract, 10g NaCl made up to 1 litre pH corrected to 7.0) without ampicillin and placed for 1 hour at 37°C in an agitator at 240 RPM. The cells were then plated (500 µl) on LB agar plates with ampicillin (50 ug/ml) and left to incubate overnight at 37°C. Colonies were then picked from the plate at 12 hours and 6 colonies were placed on a master plate (Figure 2.17). The selected clones were also

grown in 2.5 ml of LB media containing ampicillin (50 µg/ml) using an oscillation of 240 RPM in a bacterial incubator at 37° C.

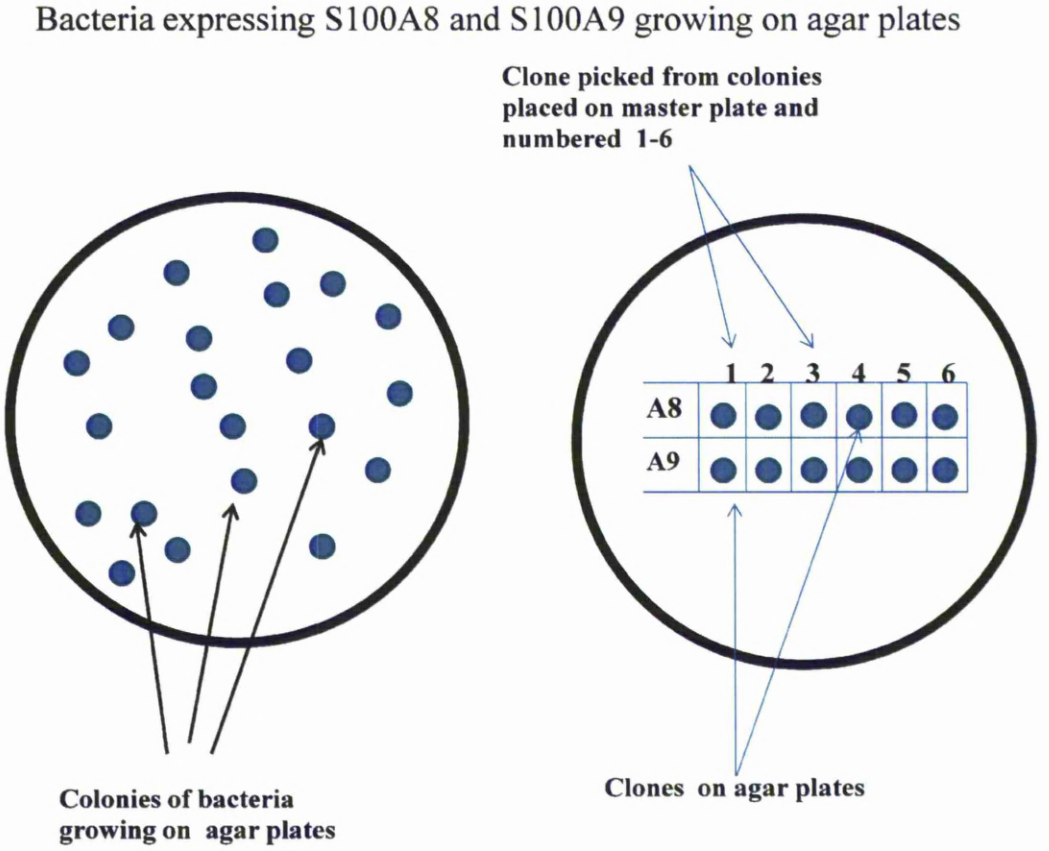


Figure 2.17: Figure showing bacteria growing on an agar plate (left) and transfer on to a master plate (right).

Plasmid DNA Purification

The QIAprep Miniprep system was used to purify DNA vectors from bacterial cells using silica membrane technology to elute high-quality plasmid DNA in a small volume of Tris buffer. The bacterial cells were lysed in an alkaline medium followed by adsorption of

DNA onto silica. The kit comes ready prepared with all reagents named. The process consists of 3 keys steps:

1. Preparation and clearing of a bacterial lysate
2. Adsorption of DNA onto the QIAprep membrane
3. Washing and elution of plasmid DNA

The protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1–5 ml overnight cultures of E.coli in LB (Luria-Bertani) medium. For these purposes colonies were picked from the master plate which contained 6 *E.coli* clones harbouring the pGEX4T-1 S100A8 vector and 6 clones with the pGEX4T-1-S100A9 vector. These cells were grown in LB media overnight and harvested by centrifugation at 6000 RCF for 3 minutes at room temperature. The supernatant was then discarded and the pellet was suspended in 250 μl buffer provided with the kit. Reagents provided in the QIA prep Miniprep system kit were then added according to manufactures protocol and the eluted DNA plasmids collected were stored at either -20°C long term or in the fridge overnight.

Restriction analysis of DNA

As mentioned above competent *E.coli* BL21 and XL1blue were used for the generation of S100A8-GST and S100A9-GST respectively. The coding sequence of S100A8 and S100A9 was cloned into the pGEX4T-1 vector using different restriction sites (for S100A8 it was EcoRI and S100A9 it was BamHI and XhoI). The pGEX4T-1 vector has a

protease cleavage recognition site for thrombin. The map of the glutathione S-transferase fusion vectors showing the reading frames and main features is shown in Figure 2.18

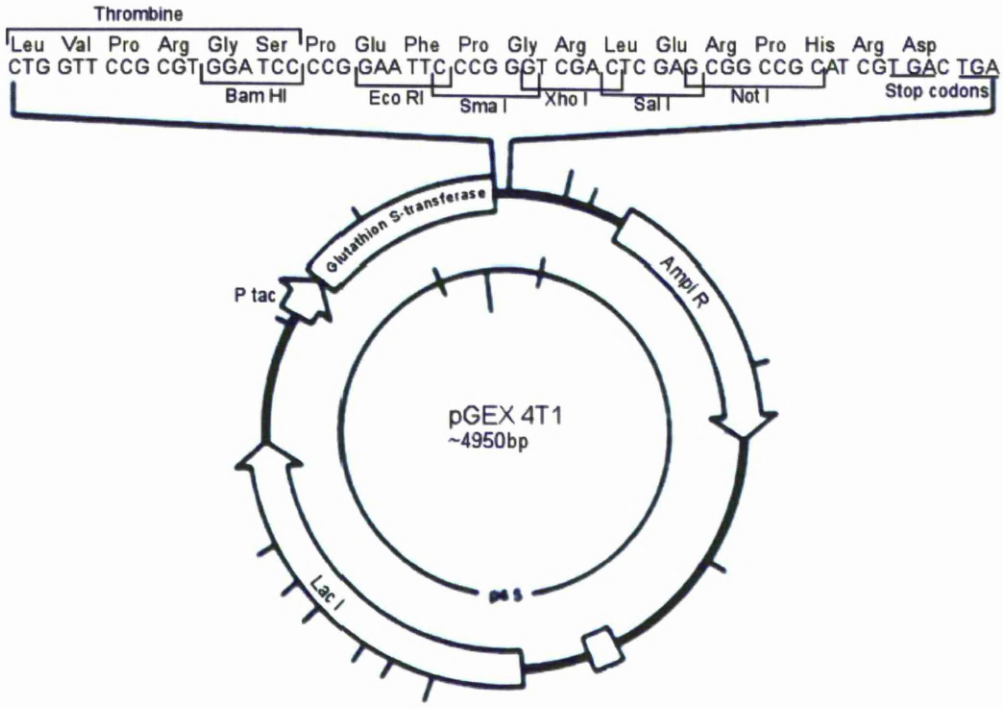


Figure 2.18: Map of the glutathione S-transferase fusion vectors showing the reading frames

Restriction analysis of DNA was undertaken by using 5µl of plasmid mini-preparation DNA and treating it with 0.3µL (3 units) of EcoRI (restriction enzyme) and 1µL O^+ buffer (50 nM Tris pH 7.5, 10 mM Magnesium Chloride, 100 mM Sodium Chloride, 0.1 mg/ml Bovine serum albumin, Fermentas) in the case of S100A8. For restriction analysis of DNA containing the S100A9 genetic sequence, 0.3µL (3 units) of XhoI buffer (10 mM Tris pH 8.5, 10 mM Magnesium Chloride, 100 mM Potassium Chloride and 0.1 mg/ml

bovine serum albumin), 0.3 µL (3 units) of BamHI (restriction enzyme) and 1µL of Y^{+}Tango buffer (33 mM Tris pH 7.9,10 mM Magnsium acetate, 66 mM Potassium acetate and 0.1 mg/ml bovine serum albumin Fermentas) were used in a 10µL reaction.

The mixture of enzymes and eluted DNA (6 clones each protein were tested) were incubated for 1 hour at 37° C. Three micro litres of 6X loading buffer (2.5 % Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017 % SDS, 0.015 % Bromophenol Blue pH 8) was added to each sample prior to loading on a 1.8% agarose gel (3.6 grams agarose, 1µL eithium bromide, 200 ml of TAE buffer[20mM Tris, 1 mL Acetic Acid, 10mM EDTA]) with a 1 kb DNA ladder and a 100 base pair ladder at either ends. Gels were electrophoresed at 100 volts for 40 minutes prior to being viewed and photographed under UV light. Colonies which did not incorporate the S100 plasmid were discarded and the rest of the colonies were picked from the master plate and cultured in LB media to make glycerol stocks (600 µL of sample and 400 µL of glycerol), that were subsequently stored at -80° C.

10.2 Generation of GST, S100A8-GST and S100A9-GST proteins

The Glutathione S-transferase (GST) gene fusion system was used for purification of fusion proteins produced in *E. Coli.* The induction of GST fusion proteins was undertaken using one clone each expressing S100A8 and S100A9 protein. The GST

control recombinant protein was a kind gift from Dr Timothy Devilling (Dr M Boyd's Lab, Division of Surgery and Oncology, School of Cancer Studies). Bacterial cells harbouring S100A8-GST or S100A9-GSTor GST were cultured in LB media by adding 100μL of cells from glycerol stocks into 1 mL culture medium and placing it at agitation (RMP 240) for 6 hours at 37°C. This culture was then diluted to larger volume of LB media (80 mL) and placed in agitation (RMP 240) overnight at 37°C. Following overnight culture cells were placed in 1 litre of LB media and allowed to culture as before. An hourly measurement of the optical density of the media was undertaken photometrically until it reached 0.8 at 600 ηm. This was undertaken to determine the optimum level of growth of *E. coli* in culture, which can be divided into distinct phases, **Lag phase, logarithmic (log) phase** and the **stationary phase.** The lag phase occurs after dilution of the starter culture into fresh medium with cell division being slow as the bacteria adapt to the fresh medium. After 4–5 hours, the culture enters the **logarithmic (log) phase,** where the bacteria grow exponentially. This phase is followed by the **stationary phase** (~16 hours) when the available nutrients are used up and bacteria begin to lyse.

Attainment of the log phase was determined when the optical density (OD) was 0.8 at 600 ηm. At that stage Isopropyl-b-D-thiogalactopyranoside (IPTG)(VWR international, Lutterworth, UK) was added to a final concentration of 0.5 mM and cultures incubated for a further 4 hours. For the generation of S100A8 and S100A9 the optimum time for expression and harvesting the bacteria was 4 hours post IPTG addition.

Purification of recombinant protein using GST fusion system

The *E.coli* cultures expressing the GST tagged proteins were collected in sterile 250 mL bottles and spun at 1000 RCF for 8 minutes at 4°C. The supernatant was discarded and the GST fusion proteins were extracted by lysing bacteria in SLIP buffer (50mM HEPES, 10% v/v glycerol, 0.1% w/v TRITON and 150mM Sodium Chloride). The cells were sheared using sonication at 20% strength for 60 seconds. Confirmation of adequate lysis was obtained by microscopy. The lysate was then spun at 4°C for 5 minutes at 500g and the supernatant collected for purification.

Glutathione sepharose beads (GE Healthcare UK and Amersham biosciences) were used to isolate the GST moiety tagged protein (S100A8, S100A9 and control GST) from the lysate using disposable columns (Bio-Rad). Glutathione Sepharose 4B, beads were initially washed in cold (4 °C) PBS, twice and 1 mL of beads was mixed with 100 mL of bacterial sonicate and incubated for 1 hour at 4°C with gentle agitation. This mixture was then centrifuged at 500g at 4°C for 5 minutes to separate the beads form the lysate. Supernatants were stored in case of unsatisfactory extraction.

The Glutathione sepharose beads were then loaded on a column (Figure 2.19) and washed with 1mL of ice cold PBS, 3 times. The wash from the column was collected for analysis. column and incubated at room temperature for 10 minutes. The outlet was then opened and the eluted protein collected. The cap was then placed on the column and a further 1mL of elution buffer added repeating the process mentioned above. The elute was then pooled and analysed, subsequently the column outlet was capped and 1 mL of elution buffer was added to the or the presence of protein.

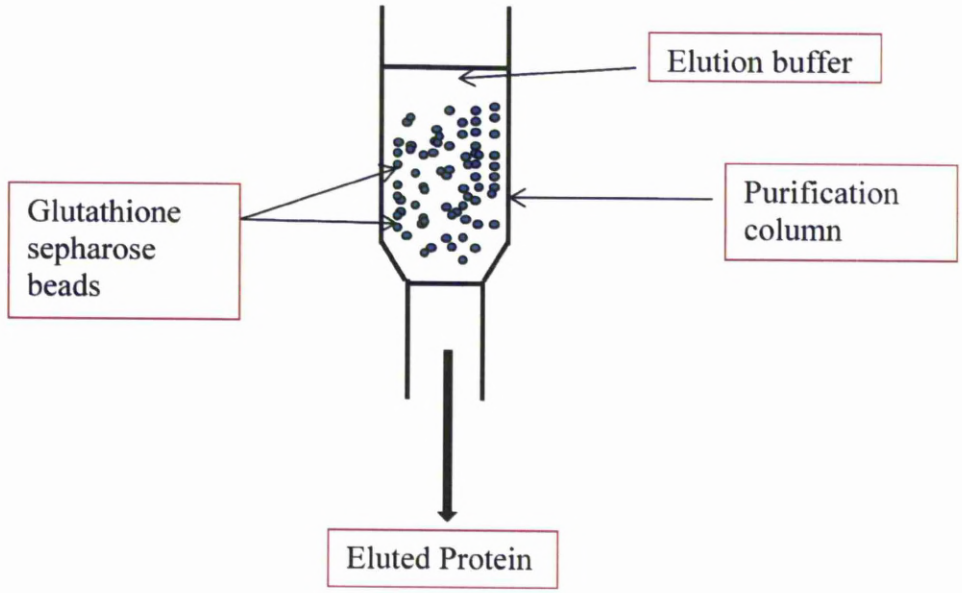


Figure 2.19: Schematic diagram showing the process of protein elution and purification using Glutathione sepharose beads on a column

Quantification of recombinant protein using Albumin standards

The quantity of recombinant S100A8-GST and S100A9-GST protein was assessed by separation on a 15% Tris-tricine SDS-PAGE gel against serial dilutions of a reference standard of BSA for comparison (1-15μg/lane). The gels were electrophoresed for 3 hours at 80 volts to allow for good resolution of the protein band. Gels were then stained by incubation overnight at room temperature with Coomassie stain solution (2.5g Coomassie blue G, 450 mL Methanol, 100 mL Acetic Acid 450 mL water) with gentle

agitation. The gels were then de-stained using a de-stain buffer (300 mL methanol, 100 mL Acetic Acid and 600 mL water) to visualise the presence of stained bands.

Densitometric evaluation of the Coomassie-stained gels (GS-800 scanner, Biorad, UK) using Quantity One software Biorad, UK) was used to quantify the concentration of protein. Three batches of proteins were generated and used in experiments to confirm reproducibility of results.

10.3 Transformation of competent cells

Plasmids encoding S100A8-GST and S100A9-GST fusion proteins in the pGEX4T-1 vector were transformed into competent *E. coli* BL21 and *E. coli* XL1blue for generation of S100A8-GST and S100A9-GST respectively. Initially the S100A9-GST vector was also transformed into *E. coli* XL1blue, however due to an additional GST tagged 26-kDa contaminant protein that was eluted along with the S100A9-GST protein, the vector was transformed into *E. coli* BL21. Subsequent to successful transformation, mini-preparation of the vector and restriction analysis of the DNA, on agarose gels was undertaken (Figure 2.20 and Figure 2.21).

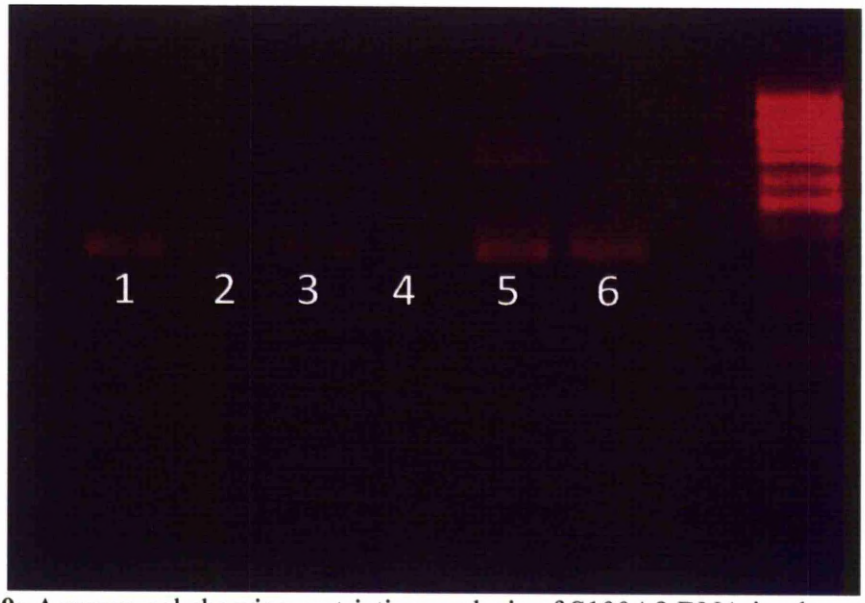


Figure 2.20: Agarose gel showing restriction analysis of S100A8 DNA in clones 1-6 in *E. coli E. coli* XL blue. Of note clone 4 shows poor uptake of DNA with strong bands in clones 1,2,3,5 and 6

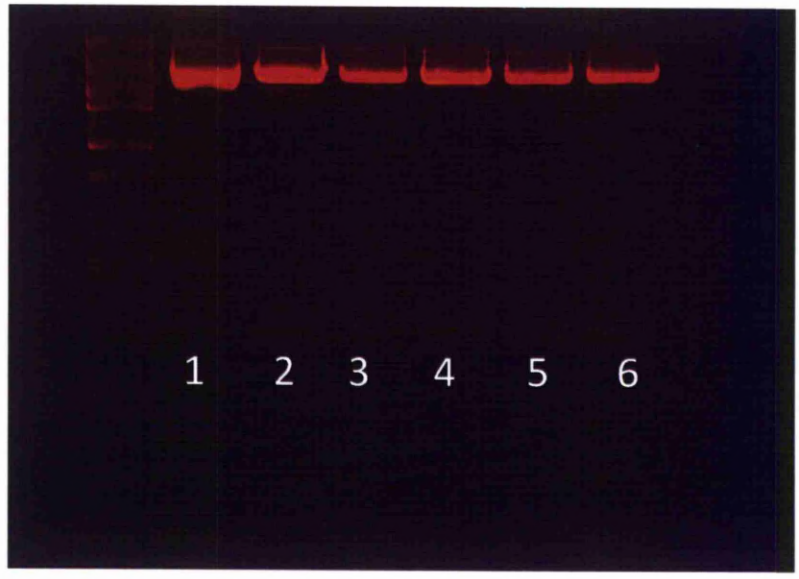


Figure 2.21: Agarose gel showing restriction analysis of S100A9 DNA in clones 1-6 in *E. coli* BL21. Of note clone 1 shows poor uptake of DNA with strong bands in clones 2-6

Purification and quantification of recombinant protein using GST fusion system

Recombinant S100A8-GST and S100A-GST9 were purified and isolated using Glutathione sepharose beads and their presence was determined on a coomassie-stained gels illustrated in Figure 2.22, where S100A8-GST is a 34 kDa band and S100A9-GST is a 40 kDa band. Densitometric evaluation of Commassie stained gels to known concentration of albumin standards, were used to quantify the proteins.

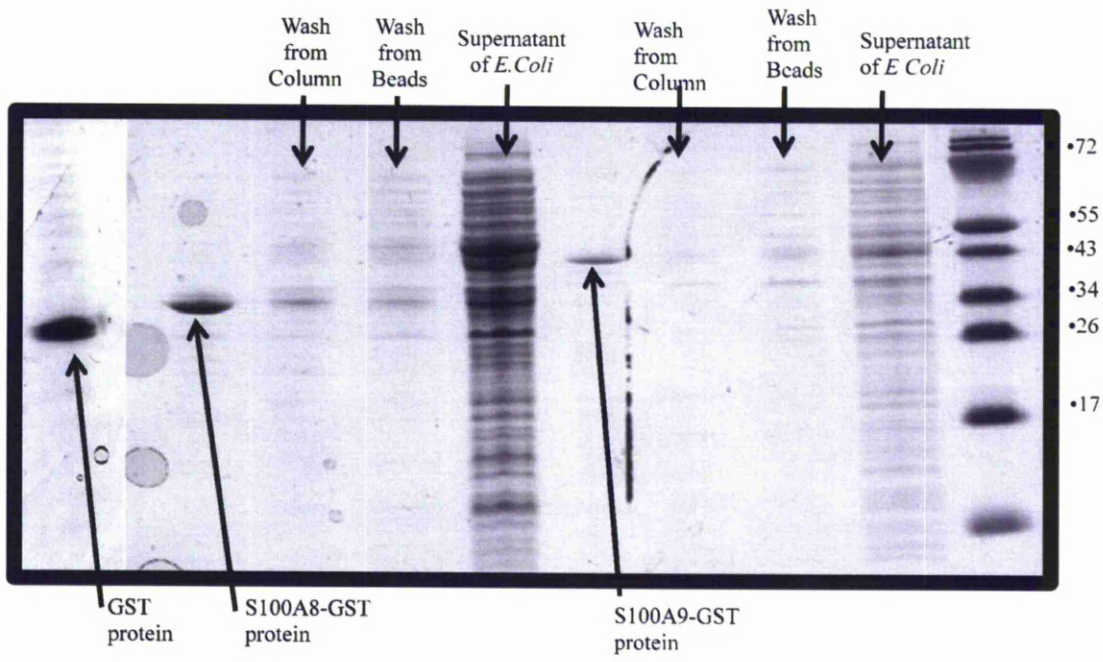


Figure 2.22: Commassie stained gel showing the presence of GST, S100A8-GST and S100A9-GST recombinant proteins and wash from glutathione column.

10.4 Generation of S100A9-GST protein using alternative methods

The plasmid for S100A9 was initially transfected in *E. coli* XL1 blue competent cells. Lysate from bacteria grown over a time course of 4 hours shows increasing presence of S100A9-GST protein being expressed over 4 hours on the 1D SDS-PAGE gel (commassie stained) (Figure 2.23).

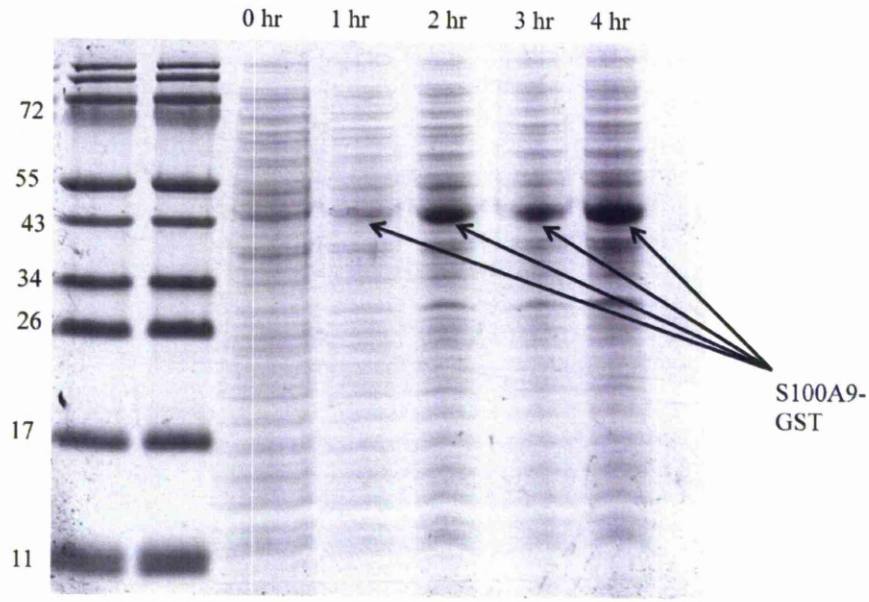


Figure 2.23: Commassie stained gel showing the presence of S100A9-GST in *E. coli* XL1blue cell lysate over 4-hour time course following initiation of induction

However, when incubated with the glutathione sepharose beads and purified on the purification column two proteins were eluted. The first was a 44 kDa protein (S100A9-GST) which was in very low quantity and a smaller 26 kDa protein, which was

selectively eluted and present in a much higher quantity (Figure 2.24). This represented an incomplete translational product of S100A9-GSTor a degraded form of the protein.

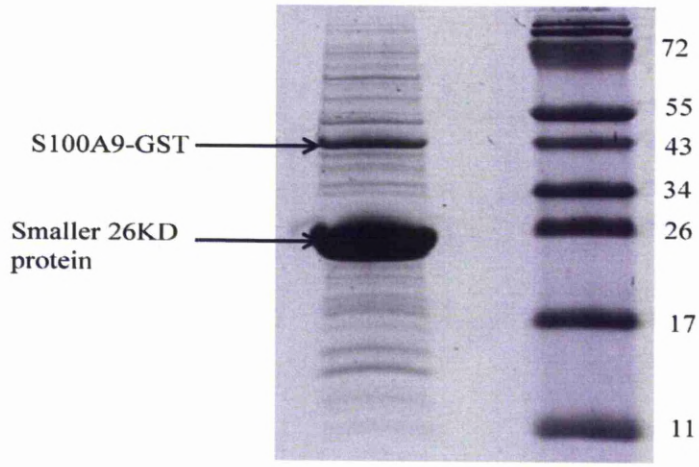


Figure 2.24: Commassie stained gel showing the presence of eluded S100A9-GST (44 kDa band) and the smaller 26 kDa protein, produced in *E. coli* XL1blue cells

In order to overcome this problem different techniques were therefore employed. Firstly, different clones were used to generate S100A9-GST protein however, they resulted in exactly the same problem as described above. Secondly, elution of protein on the separation column was undertaken at 4°C, Room temperature and at 37 °C, however as shown in the Commassie stained gels of protein eluted using sepharose beads no difference was achieved and two bands were identified one at 44 kDa and the other at 26 kDa. Thirdly, I grew the bacteria in special culture media, hypermedia (Sigma-Aldrich,UK) and powermedia (Sigma-Aldrich, UK) as opposed to standard LB media at room temperature and 37 °C. These adaptations did not resolve the issue and the contaminant protein was still present. Therefore, the S100A9 plasmid was transfected in

the *E. coli* BL21 codon competent cells which alleviated the problem as shown in the Commassie stained gel below (Figure 2.25).

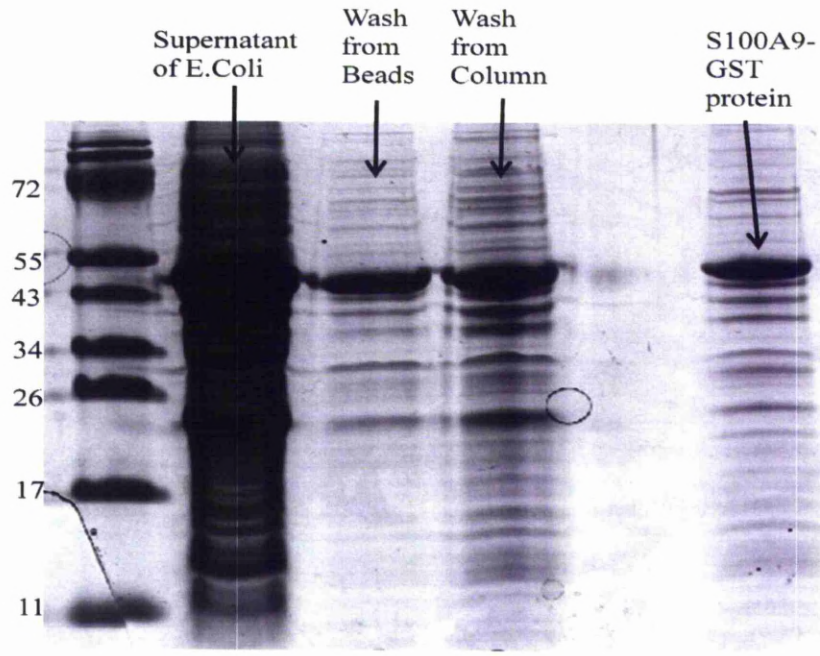


Figure 2.25: Commassie stained gel showing the expression of S100A9-GST produced in *E. coli* BL21 cells and eluted on a glutathione column.

10.5 Discussion

Cell culturing experimentation using conditioned media to treat primary monocytes and monocytic cell lines resulted in increased expression of S100A8 and S100A9 proteins in monocytes, suggesting a potential crosstalk channels between the 2 cells in the pancreatic cancer microenvironment. To further elucidate this, recombinant GST tagged proteins where generated in the Glutathione S-transferase (GST) gene fusion system. This gene fusion system is a versatile system for the expression, purification, and detection of fusion proteins produced in *E. coli.* Expression in *E. coli* yields fusion S100A8 and S100A9 proteins with the GST moiety at the amino terminus and the S100 protein at the carboxyl terminus. One of the potential advantages of GST fusion proteins is the control under the *TAC* promoter, which is induced by the lactose analog isopropyl b-D thiogalactoside (IPTG). All pGEX vectors are engineered with an internal *lac*Iq gene. The *lac*Iq gene product is a protein that binds to the *TAC* promoter, preventing expression until induction by IPTG, therefore maintaining a chemically inducible control over expression. Potential disadvantages of using the GST-fusion systems are the production of oligomers via the GST moiety and hindrance caused by the large fusion tag. In addition, the binding of the GST moiety can result in inadequate folding of the primary protein affecting its functional capacity. The GST tagged S100A8 and S100A9 which were produced for experimentation purposes have been used previously and were generated in a similar manner (Hiratsuka et al., 2006).

The generation of S100A8-GST did not produce any significant problem with the protein being generated and eluted as expected. The generation of S100A9 however produced

significant problems when the plasmid was transfected into *E. coli* XL1blue. The resultant 26KDa protein is likely to have been an incomplete translational product of S100A9-GST or a degraded form of the protein. The incomplete translational product produced may have been a result of a process called codon bias (Ermolaeva, 2001). A codon is a series of three nucleotides (triplets) that encodes a specific amino acid residue in a polypeptide chain or amino acids for the termination of translation (stop codons). Even though there are 64 different codons (61 codons encoding for amino acids plus 3 stop codons) only 20 are translated into amino acids. In this process, the translation of the recombinant RNA is delayed, resulting in degraded RNA or codon substitutions and mis-incorporations that destroy the functional characteristics of the protein or result in a protein, which is not a full length sequence. Different experimental conditions applied did not resolve this problem and therefore, the S100A9 plasmid was transfected in the *E. coli* BL21 codon plus competent cells. The BL21-Codon Plus cells (Jerpseth et al., 1998) are designed with extra copies of tRNA genes that are rare in *E. coli* but frequently used in humans. These have been shown to prevent codon bias from occurring and allowing for high-level expression of proteins that are difficult to express in conventional *E. coli* hosts.

CHAPTER 11

IN-VITRO CELL MOTILITY AND PROLIFERATION EXPERIMENTS USING RECOMBINANT GST TAGGED S100A8 AND S100A9

11.1 Cell motility measurement – The modified Boyden chamber assay

A modified Boyden Chamber assay (Figure 2.26) was used to assess the motility of pancreatic and colorectal cancer cell lines (Thompson et al., 2007). A cell culture insert with PET membrane transwell 8μm pores (VWR) was placed into each well of a 24-well plate as an upper chamber. Cells of interest were harvested and plated in 500μl of serum-free medium in the upper chamber whereas the lower chamber contained 750μl of cell culture media with varying conditions.

Recombinant S100A8-GST and S100A9-GST at concentrations of 0.4 μg/ml and 2 μg/ml were prepared in culture media containing 1% FCS, and placed in the lower chamber either singly or in combination. Controls for this experiment were culture media containing 1% FCS with GST protein, 10% FCS, or 1% FCS alone. The chambers were incubated at 37°C for 18 hours, at which time, non-migrating cells were removed from the upper surface of inserts with a cotton swab. Cells which migrated through the pores to the under-surface of the insert membrane, were fixed and stained using Diff-Quick Stain Kit (Reagena). The membrane was then cut out and mounted onto glass slides and stained cells were counted with a microscope at 40x magnification. Duplicate or triplicate inserts were set up for each condition and the experiments were carried out on at least on two separate occasions.

The number of cells placed in the upper chamber varied depending on the base line motility of the cells. Colorectal cell lines (SW-480/SWD20/SWK3/SWD14/SWD1/ and SW-837) were plated at a density of 5 x 10^4 cells whereas pancreatic cancer cells PANC-, Suit-2 and MIA PaCa-2 cells were plated at a density of 5 x 10^3 cells in the upper chamber.

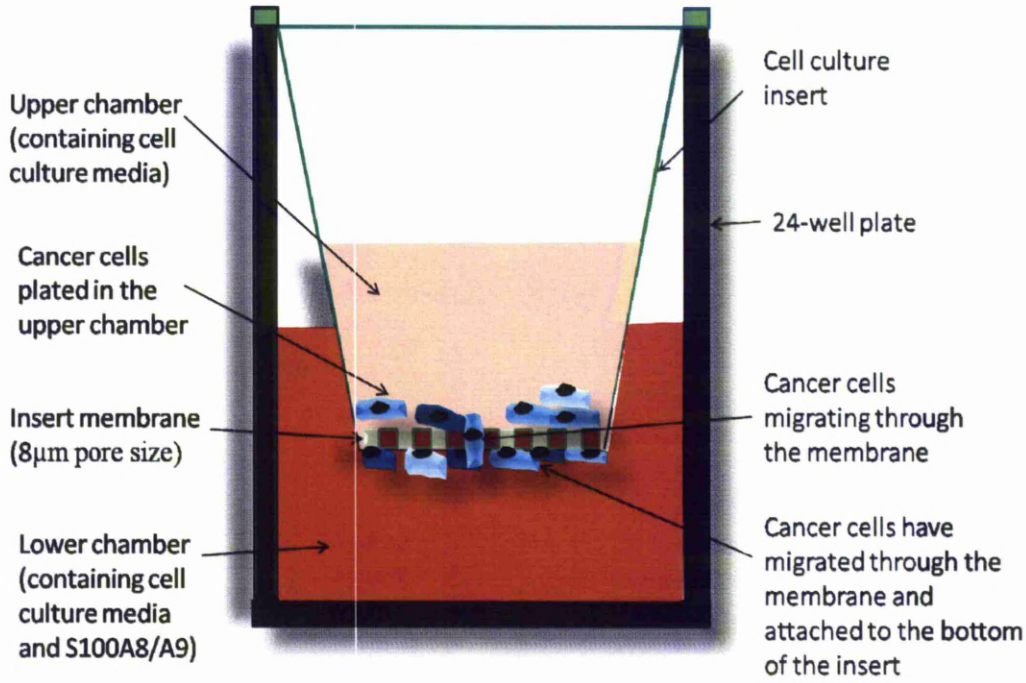


Figure 2.26: Figure showing a Boyden chamber and various components of the experimental setup

11.2 Cell proliferation assay

Proliferation of colorectal and pancreatic cancer cells were assessed at 24 h, 36 h and 48 h using a MTT assay utilizing the EZ4U non-radioactive cell proliferation assay (Biomedica, Vienna) according to the manufacturer's instructions.

Cells were freshly suspended in 1% FBS culture media at a concentration of $3x10^4$ cells/mL. One hundred micro litres of cell suspension was added to 96-well plates containing 100μL of 1% FBS media with recombinant S100A8-GST, S100A9-GST or GST at 0.4μg/mL and 2μg/mL concentrations. The S100 proteins were either added singly or in combination. Experiments were performed three times and the number of wells utilised for each condition was either 12 or 6. Readings at absorbance of 450 nm were taken at 4h following incubation with the reagents. Experiments were performed at least three times and five wells utilised for each treatment.

11.3 Transient SMAD4 knockdown methodology

Pancreatic and colorectal cancer cells transiently depleted of SMAD4 were generated to assess differential effects of treatment with S100A8 and S100A9 proteins in terms of motility and proliferation. Transient knockdown of SMAD4 was undertaken in SWD20 and PANC-1 cells using siRNA molecule sequences GUGUGCAGUUGGAAUGUAA (siRNA1, Dharmacon) and GUACAGAGUUACUACUUAG (siRNA2, Dharmacon). Two control siRNA's were used:

1) Non-targeting siRNA control (Dharmacon)
2) Scrambled siRNA control (GGACGCAUCCUUCUUAA, a gift from Dr M Boyd, University of Liverpool, UK).

Once transfected, the cells were harvested at 48 hrs which was deemed the optimum time for knockdown. This was determined by serial experiments assessing knockdown at 24h, 48h and 72h.

Transient SMAD4 depletion/knockdown

Cells were harvested, washed, counted, and plated into 6-well plates at a density of $1x10^5$ cells in each well to achieve a cell density of ~60-70%. Approximately 3 ml of culture media was added into each well and the plate was incubated at 37°C for 24 hours.

For transfection of each well, 4μL of lipofectamine 2000 (Sigma UK) was added to 200μL of Optimem I (Sigma UK) in one 15 mL tube, and siRNA (ranging from 5-50 nM) was added to 200μL of Optimem I in another 15mL tube. These two tubes were incubated for 5 minutes in the laminar flow hood before mixing their contents together and incubating for a further 20 min.

The 6-well plates were retrieved from the incubator, the wells washed with PBS and 2.6 ml of antibiotic-free culture media was added into each well. The siRNA and lipofectamine 2000 mixture was then added to the wells in a drop-wise manner. The plates were returned to the incubator for 48 hours and cells harvested for further experiments.

11.4. Cell motility experiments

The effects of S100A8-GST and S100A9-GST on Pancreatic cancer cell motility

Pancreatic cancer cells (PANC-1, SUIT-2 and MIAPaCa3) were treated with recombinant S100A8-GST and S100A9 -GST, which induced increased motility of these cell lines. All cell lines responded to both low (0.4μg/mL) and high (2μg/mL) concentrations of S100A8-GST and S100A9-GST when administered alone or in combination. **PANC-1 cells** showed a significant increase in motility compared to GST control (n=5 experiments done in triplicate), when treated with a low concentration (0.4 μg/mL) of S100A8-GST ($p<0.001$; paired t-test), S100A9-GST ($p=0.0004$; paired t-test) and combined S100A8-GST and S100A9-GST ($p=0.002$; paired t-test) recombinant proteins (Figure 2.27). Similarly a significant increase in motility was observed when these cells were treated with high concentration S100A8-GST ($p=0.0005$; paired t-test) and S100A9-GST ($p=0.0079$; paired t-test). Combined high concentration S100A8-GST and S100A9-GST ($p= 0.0161$; paired t-test) produced a dramatic increase in motility with a near doubling effect.

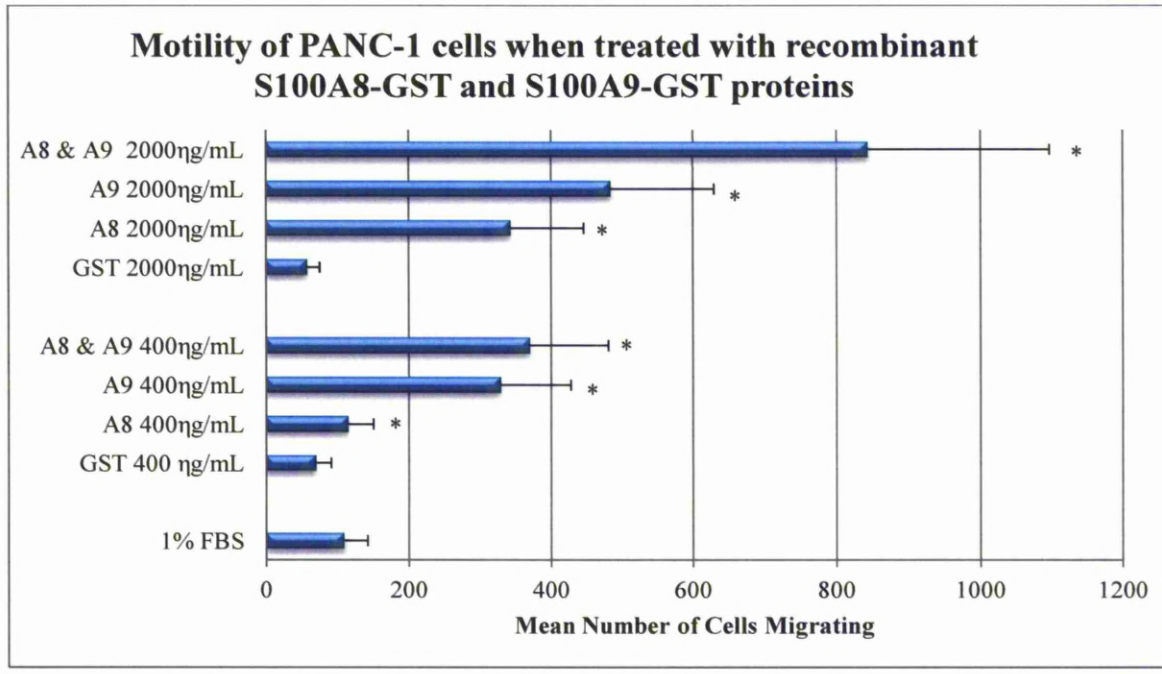


Figure 2.27: Figure showing the mean counts of migrating PANC-1 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=5). * denotes significant result

SUIT-2 cells also showed a significant increase in motility compared to respective GST control (n=3 experiments, done in triplicate) (Figure 2.28), when treated with a low concentration (0.4µg/mL) of S100A8-GST ($p=0.01$; paired t-test), S100A9-GST ($p=0.02$; paired t-test) and combined S100A8-GST and S100A9-GST ($p=0.05$; paired t-test) recombinant proteins. Similarly, a significant increase in motility was observed when these cells were treated with high concentration S100A8-GST ($p<0.01$; paired t-test) and S100A9-GST ($p=0.01$; paired t-test). Combined high concentration S100A8-GST &S100A9-GST also produced a significant increase in motility ($p= 0.05$; paired t-test).

The response of SUIT-2 cancer cell lines to low and high concentration S100A8-GST was more pronounced than that of S100A9-GST.

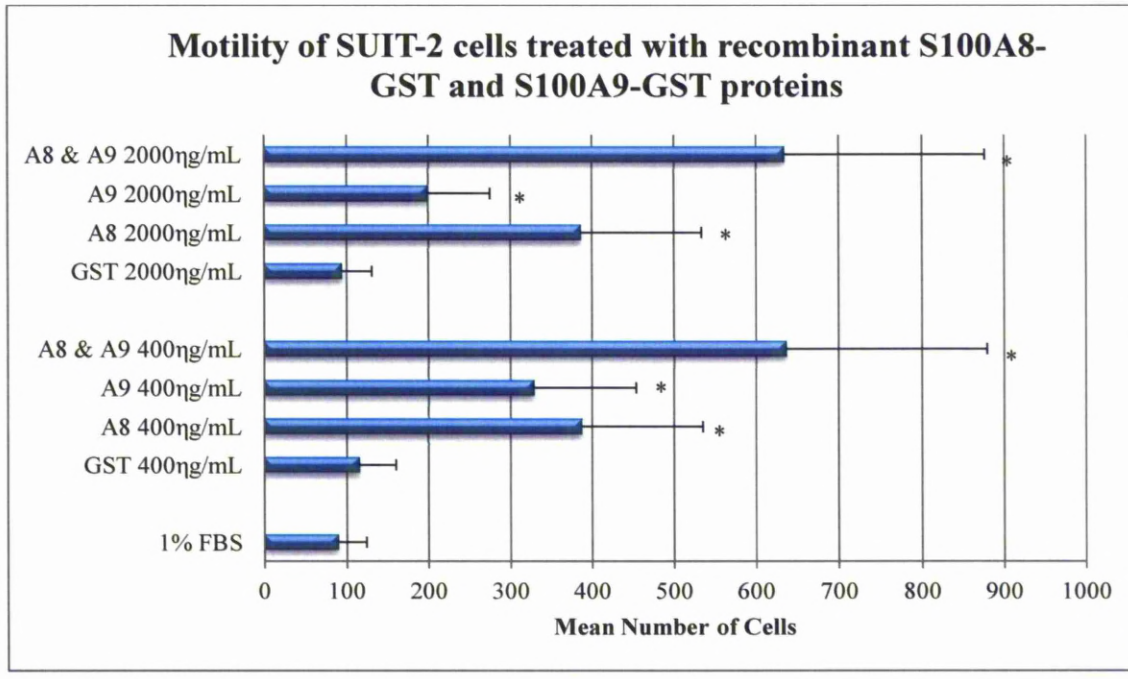


Figure 2.28: Figure showing the mean counts of migrating SUIT-2 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

MIA PaCa-2 cell lines demonstrated a significant increase in motility compared to respective GST controls (n=3 experiments, done in triplicate) (Figure 2.29), when treated with a low concentration (0.4µg/mL) of S100A8-GST (p=0.05; paired t-test) but not with high concentration of the protein (2µg/mL; paired t-test). A significant increase in

chemotaxis was achieved when the cells were treated with low concentration S100A9-GST (p=0.03; paired t-test) and combined S100A8-GST & S100A9-GST (p=0.04; paired t-test) recombinant proteins. At high concentrations of S100A9-GST and combination S100A8-GST & S100A9-GST (n=2) MIA PaCa-2 cells also produced a significant increase in motility (p=0.01; paired t-test for S100A9-GST and p=0.019; paired t-test for combined S100A8-GST and S100A9-GST).

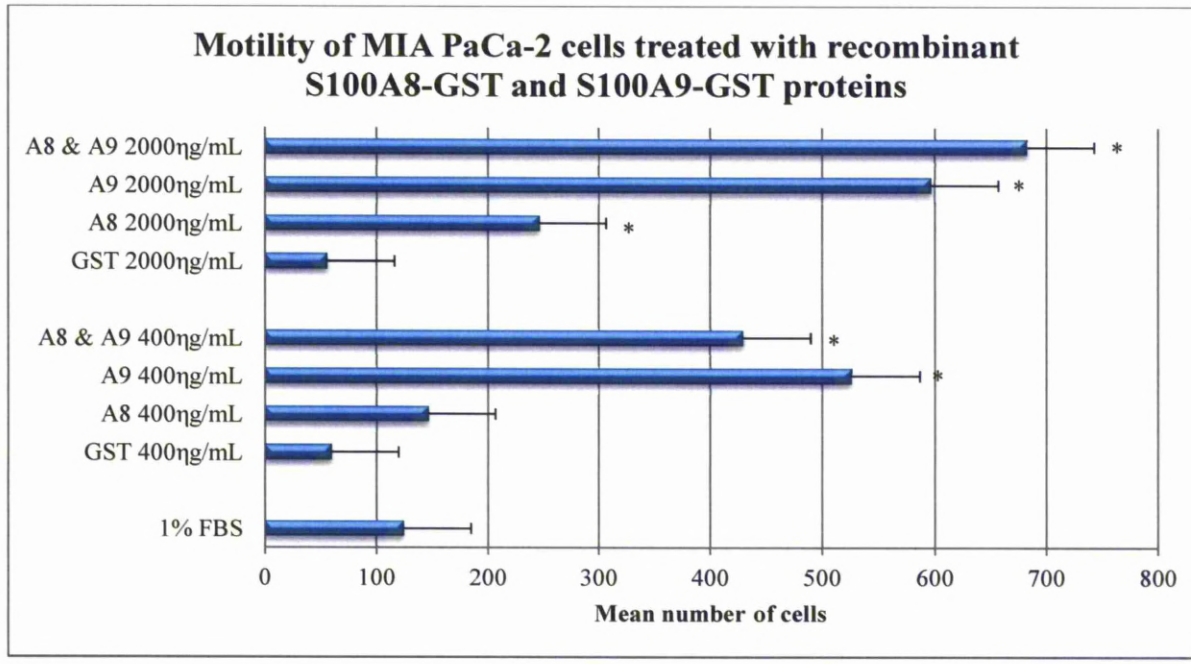


Figure 2.29: Figure showing the mean counts of migrating MIA PaCa-2 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

The effects of S100A8-GST and S100A9-GST on Colorectal cancer cell motility

Both recombinant S100A8-GST and S100A9-GST proteins induced motility of colorectal cell lines SW-837 and SW-480. **SW-480** (Figure 2.30) (n=4 experiments, done in triplicate) cells demonstrated a dramatic increase in motility following incubation with low (0.4μg/mL) and high (2μg/mL) of concentration of S100A8-GST compared to GST alone ($p=0.01$ and $p=0.001$ respectively; paired t-test). A less marked affect was observed in response to low concentration S100A9-GST with a marginal increase in chemotaxis observed at a higher concentration of S100A9-GST protein ($p=0.05$, and $p=0.004$; paired t-test respectively). Combined S100A8-GST and S100A9-GST at a low and high concentration induced a significantly appreciable increase in motility ($p=0.03$, $p<0.01$; paired t-test) however, it was not as effective as S100A8-GST alone.

The response of **SW-837** cells (n=3 experiments, done in triplicate) (Figure 2.31) was somewhat varied to both low (0.4μg/mL) and high (2μg/mL) concentrations of S100A8-GST and S100A9-GST. S100A8-GST proteins produced a significant increase in motility at high concentrations but not at low concentrations ($p=0.04$ for high concentration and 0.09 for low concentration; paired t-test). S100A9-GST produced a more pronounced effect compared to S100A8-GST in terms of motility, it only reached significance at low concentrations ($p=0.05$ paired t-test) but not at the higher concentration ($p=0.1$; paired t-test). The combination of both proteins produced significant increases in motility at both low and high concentration respectively ($p=0.01$ and $p=0.05$ respectively paired t-test).

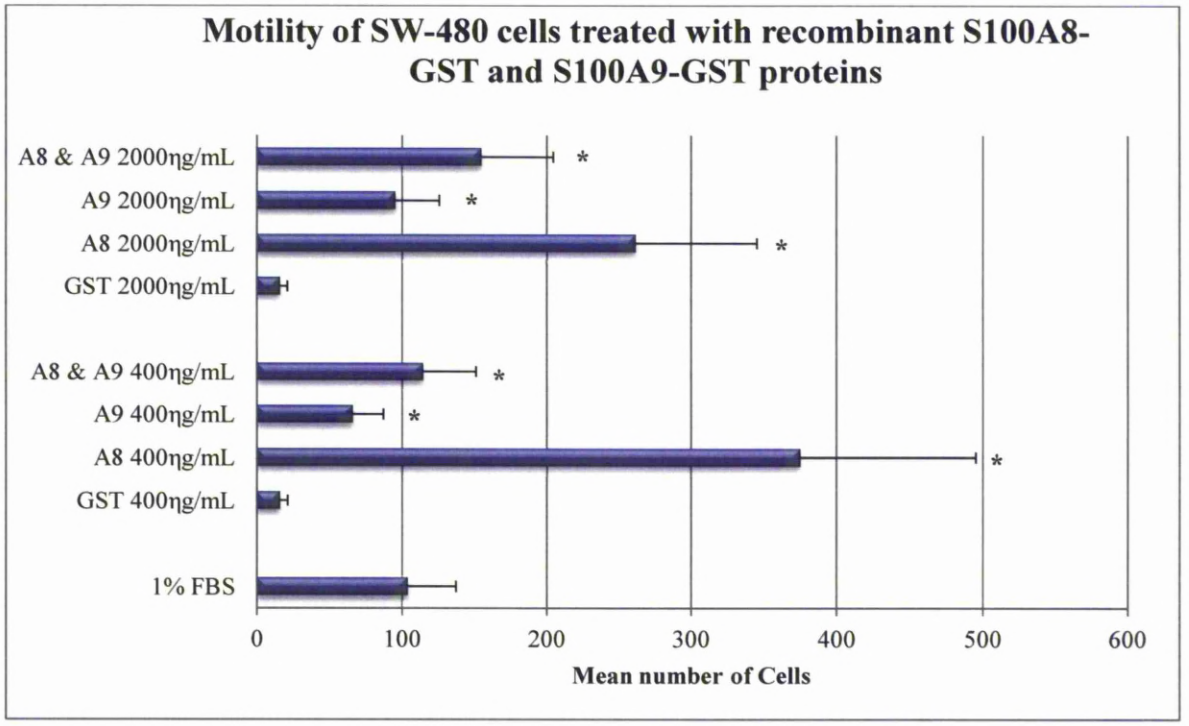


Figure 2.30: Figure showing the mean counts of migrating SW-480 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4μg/mL or 2μg/mL singly or in combination (n=4). * denotes significant result

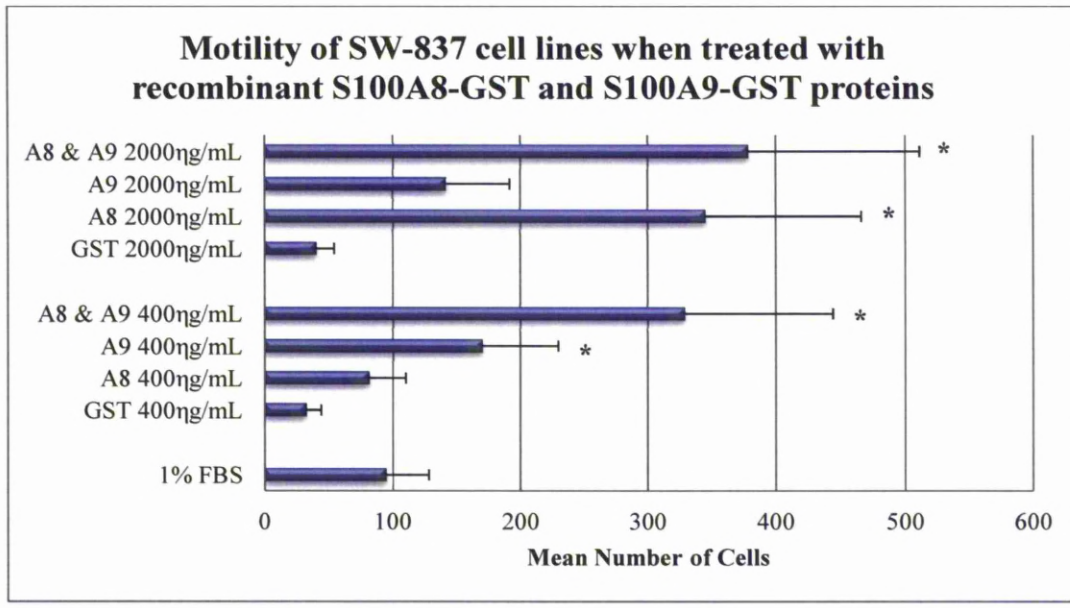


Figure 2.31: Figure showing the mean counts of migrating SW-837 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

The effects of S100A8-GST and S100A9-GST on the motility of SMAD4-positive and -negative colorectal clones

In order to evaluate any differential effects the SMAD4 status of the cancer cells may pose on the chemotactic response to S100A8-GST and S100A9-GST recombinant proteins, SMAD4 re-expressing stable clones of colorectal cancer cell lines SW480 were used. These cell lines have varied expression of SMAD4 with SWD20, SWD14 and SWD1 expressing varying amounts of SMAD4 positivity and SWK3, expressing a small amount therefore being considered as a SMAD4 negative clone.

The motility of the SMAD4 negative subclones **SWK3** (n=3 experiments done in duplicate) (Figure 2.32) significantly increased compared to GST controls when treated with low concentration (0.4µg/mL) of S100A8-GST (p=0.05; paired t-test). S100A9-GST recombinant protein at low concentration was not tested in this cell line. At the higher concentration (2µg/mL), S100A8-GST attainted a significant increase in motility (S100A8-GST p= 0.05 paired t-test) but this was not the case with S100A9-GST (p= 0.5 paired t-test). A combination of both proteins at high concentration (2µg/mL) produced significant chemotaxis (p=0.05 paired t-test) with a pronounced effect seen.

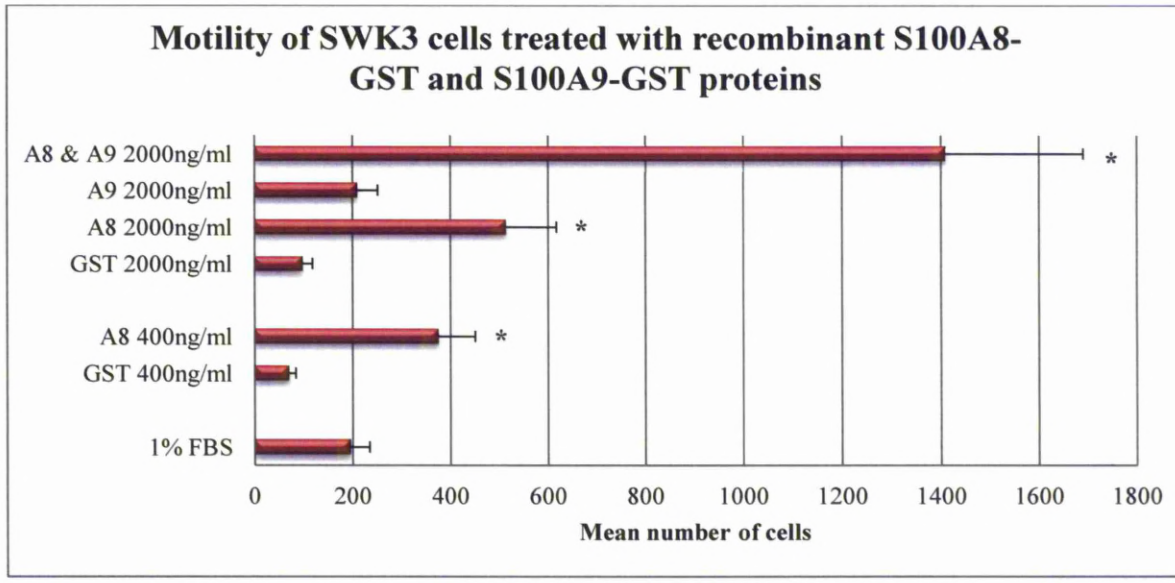


Figure 2.32: Figure showing the mean counts of migrating SWK3 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

Interestingly S100A9-GST protein did not induce motility in either **SWD1** (Figure 2.33) and **SWD14** (Figure 2.34) subclones at either a low (0.4μg/mL) (SWD1, p=0.11 and SWD14, p=0.2; paired t-test) or high concentrations (2μ/mL) (SWD1, p=0.1 and SWD14 p=0.2; paired t-test) of the protein (n=3 experiments done in duplicate). Both of these subclones however responded to recombinant S100A8-GST (0.4μg/mL) (n=2) inducing a significant increase in motility (SWD1, p=0.03; SWD14, p=0.03 paired t-test). Similarly using higher concentration of S100A8-GST (2μg/mL) induced a statically significant chemotactic response (SWD1 p=0.04; SWD14 p=0.001; paired t-test). When both proteins were applied in combination a significant increase in motility was induced both in SWD1 and SWD14 cell lines with 2μg/mL of protein (p= 0.05 and 0.009 respectively; paired t-test) as well as with a 0.4μg/mL of combined S100A8-GST and S100A9-GST (SWD1 p=0.03; SWD14 p=0.006; paired t-test).

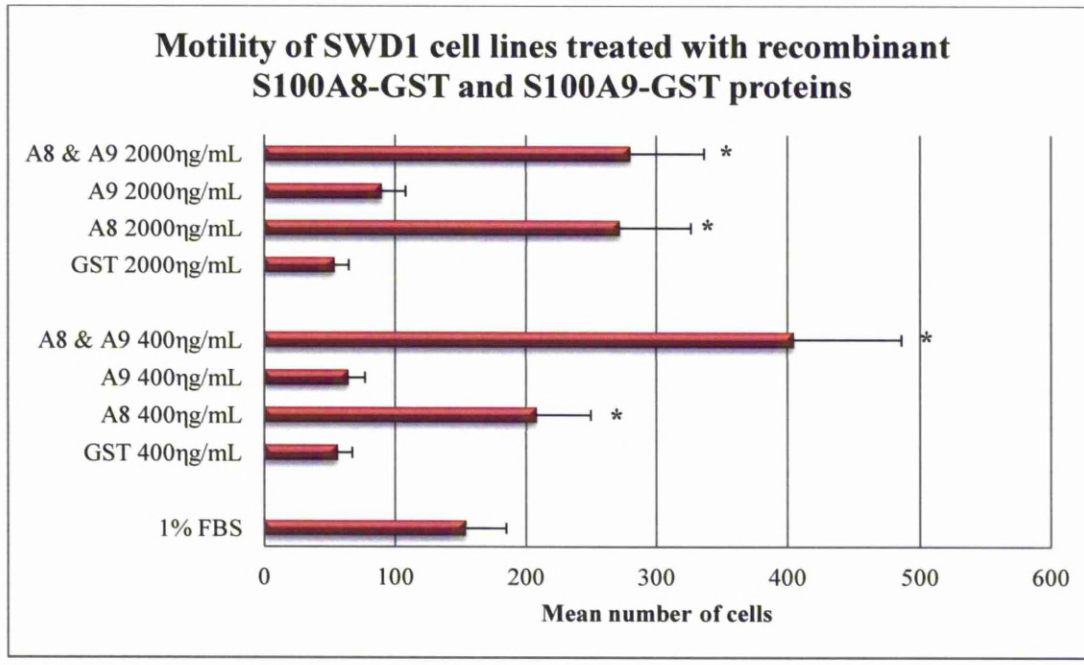


Figure 2.33: Figure showing the mean counts of migrating SWD1 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

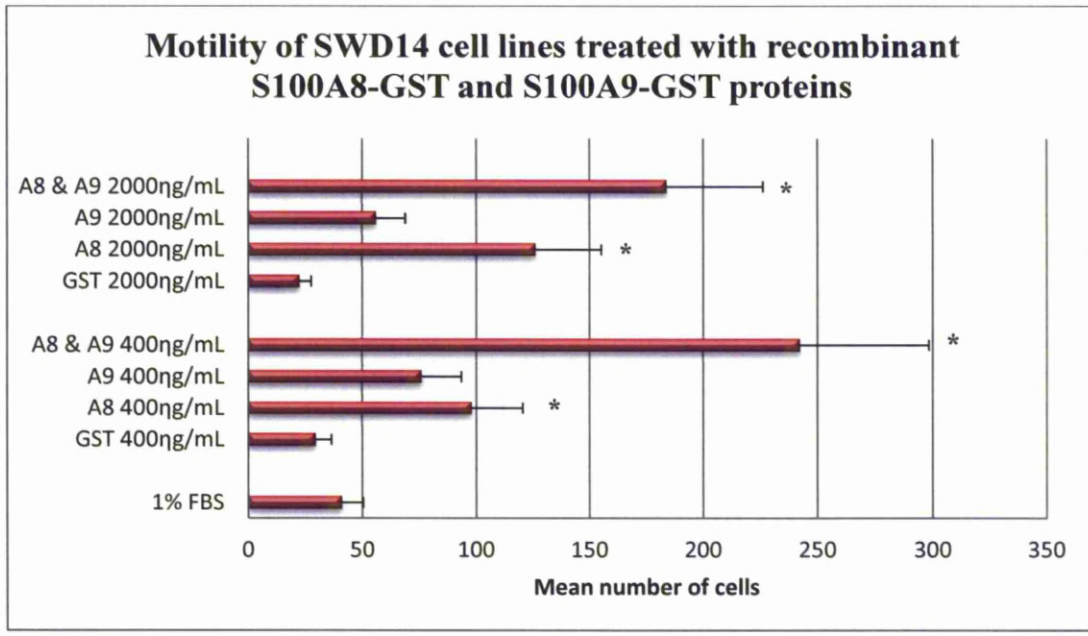


Figure 2.34: Figure showing the mean counts of migrating SWD14 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=3). * denotes significant result

The subclone **SWD20** (Figure 2.35) (n= 4, experiments done in duplicate) which in comparison to the other clones expressed the highest quantity of SMAD4, showed a positive chemotactic response to recombinant S100A8-GST and S100A9-GST when treated at concentrations of 0.4µg/mL and 2µg/mL of the protein (S100A8: 0.4µg/mL, and 2µg/mL; $p < 0.001$) (S100A9-GST: 0.4µg/mL and 2µg/mL, $p < 0.001$; paired t-test). When a combination of both proteins was applied a significant increase in motility was observed at both low and high concentrations respectively ($p<0.001$ and $p=0.02$ respectively; paired t-test).

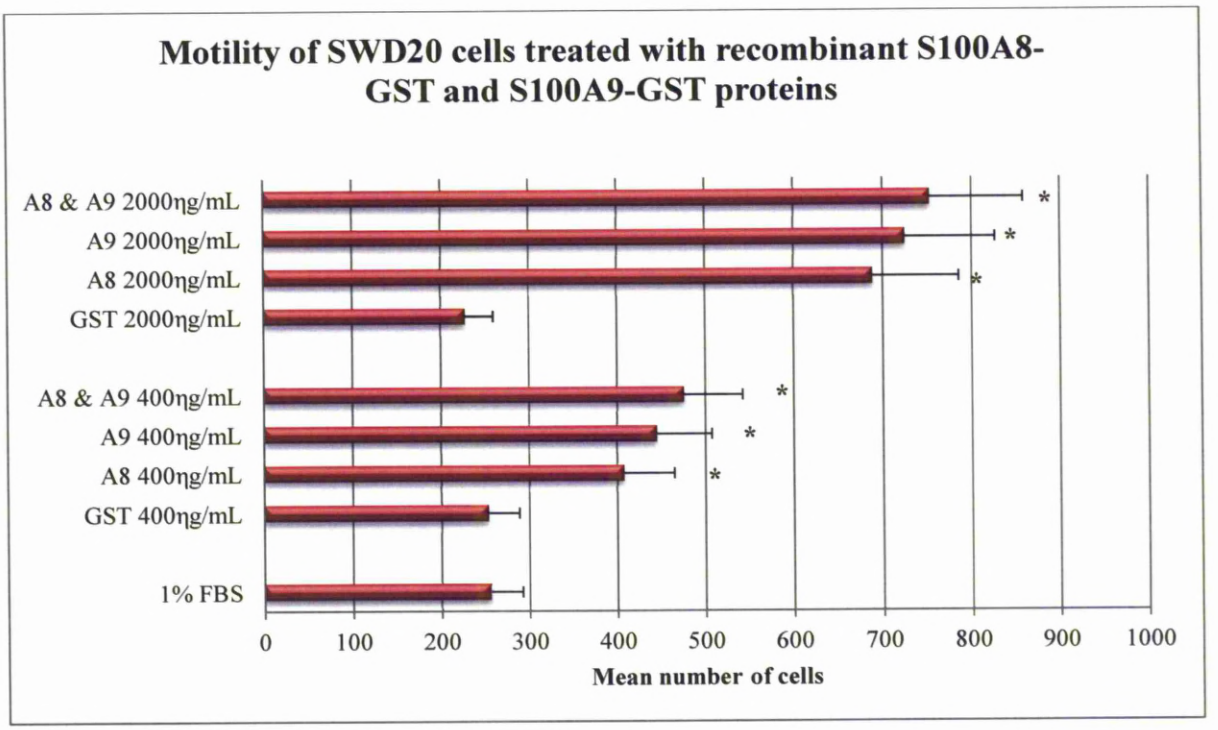


Figure 2.35: Figure showing the mean counts of migrating SWD20 cells 18h post incubation with recombinant S100A8-GST (A8) or S100A9-GST (A9) or GST at 0.4µg/mL or 2µg/mL singly or in combination (n=4). * denotes significant result

As demonstrated in the aforementioned results no consistent difference was observed in the effects S100A8-GST and S100A9-GST recombinant proteins had on the motility of colorectal sub-clones of SW-480 based on their SMAD4 status. As SMAD4 is a protein normally lost in the process of carcinogenesis it was therefore decided that producing a SMAD4 knockdown in SMAD4 positive cell lines could be a more viable method to study the effects that SMAD4 may have on the motility of the pancreatic cancer cells when treated with recombinant S100A8-GST and S100A9-GST proteins.

The effects of S100A8-GST and S100A9-GST on SMAD4 knockdown pancreatic cancer cell lines

Transient knockdown of SMAD4 in PANC-1 cell lines was achieved in 24 hours however a complete knockdown was established at 48 hours after transfection as demonstrated in the western blot analysis which shows knockdown of SMAD4 at 48 hours following treatment with two interference RNA (siRNA1 and siRNA2) (Figure 2.36).

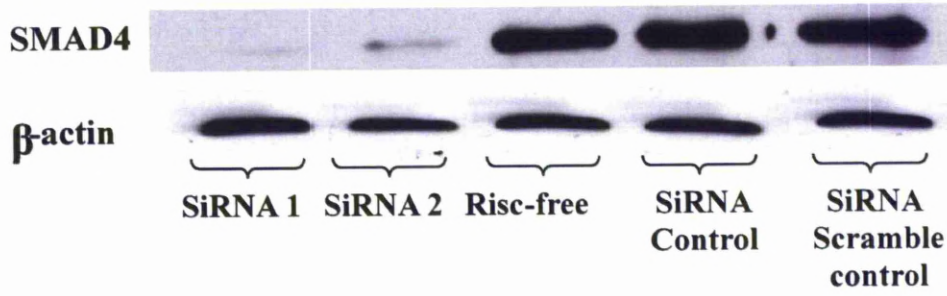


Figure 2.36: Western data showing transient knockdown of PANC-1 cancer cells and control at 48 hours post transfection with various conditions.

The SMAD4-depleted cells demonstrated an overall decrease in baseline motility compared to the controls (Figure 2.37 and Figure 2.38). When treated with recombinant S100A8-GST and S100A9-GST (singly or combined) at low (0.4 μg/mL) and high (2μg/mL) concentrations (n=2 experiments done in duplicate) cells with SMAD4 depleted by siRNA1 had a more pronounced chemotactic response in comparison to cells which were knockdown using siRNA2. Cells which were knockdown by siRNA1 (and not by siRNA2) also demonstrated a greater chemotactic response when treated with

recombinant S100A8-GST and S100A9-GST (singly or combined) at low concentration (0.4 µg/mL) compared to the scrambled control only and not to RISC-FREE and Control-1. This effect was not demonstrable when treated with higher concentrations (2µg/mL) of both recombinant proteins (applied singly or in combination). Owing to the experiment being conducted twice, no statistical calculations were possible to make a safe conclusion.

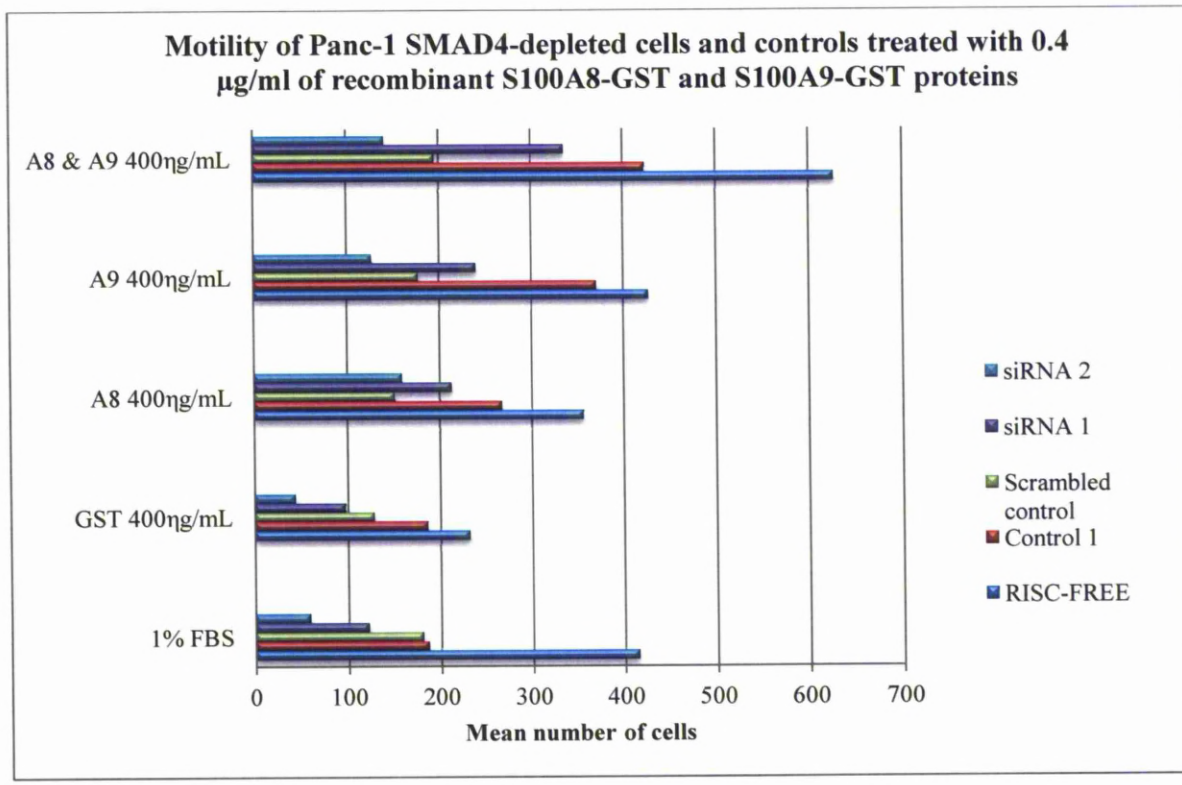


Figure 2.37: Figure showing the mean counts of migrating SMAD4-depleted PANC-1 and control PANC-1 cells, 18h post incubation with recombinant S100A8-GST or S100A9-GST or GST at 0.4µ/mL.

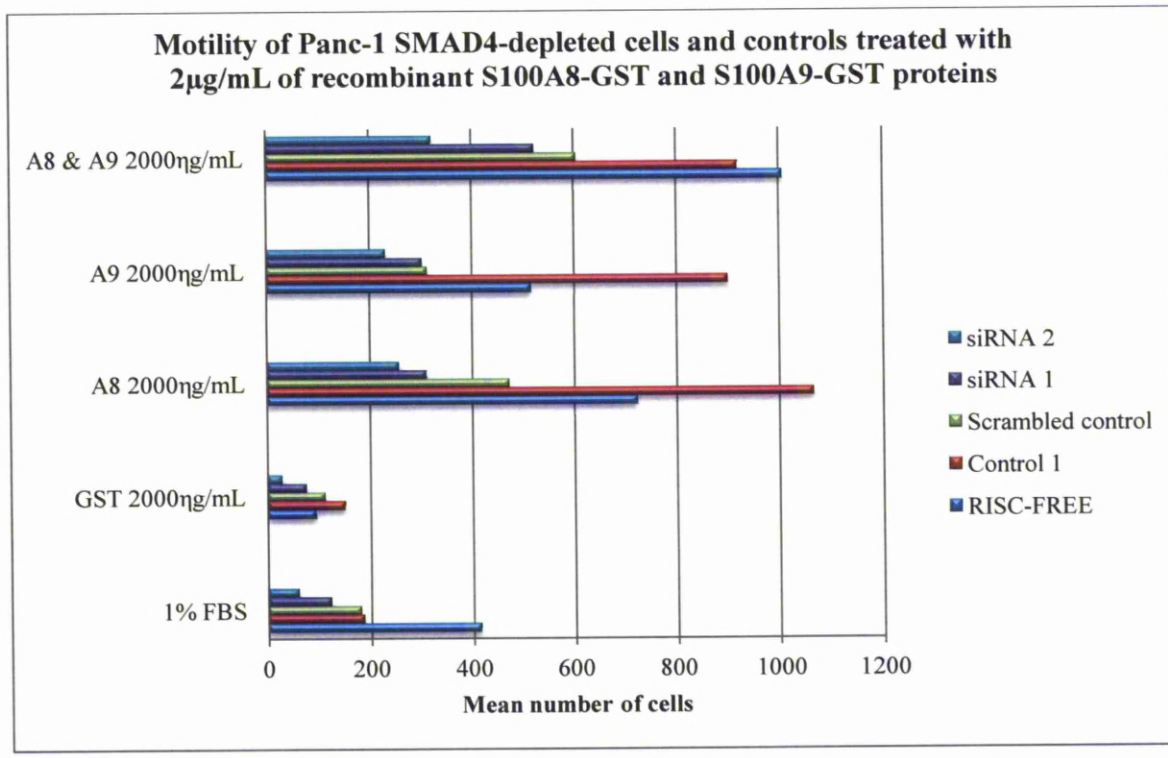


Figure 2.38: Figure showing the mean counts of migrating SMAD4-depleted PANC-1 and control PANC-1 cells, 18h post incubation with recombinant S100A8-GST or S100A9-GST or GST at 2µg/mL.

11.5 Cell proliferation experiments

The effects of S100A8-GST and S100A9-GST on cell proliferation

The proliferation of pancreatic and colorectal cancer cell as described in the previous sections was assessed following incubation with medium containing 1% FBS supplemented with the recombinant proteins S100A8-GST, S100A9-GST or control GST, generated and purified from *E. coli*.

The effects of S100A8-GST and S100A9-GST on Pancreatic cancer cell proliferation

Pancreatic cancer cells (PANC-1, SUIT-2 and MIAPACA3) were incubated with recombinant S100A8-GST and S100A9-GST and their proliferation at 24h, 36h and 48h was measured. All three of the cell lines responded to both low (0.4μg/mL) and high (2μg/mL) concentrations of S100A8-GST and S100A9-GST when administered alone or in combination.

PANC-1 cell lines showed a significant increase in proliferation at 48 hours compared to their respective GST control (n=3 experiments conducted in quintet). When treated with a low (0.4μg/mL) and high (2μg/mL) concentration of S100A8-GST ($p=0.05$ for low concentration and $p<0.001$ for high concentration; paired t-test). Similarly at 48hrs when treated with S100A9-GST a significant increase in proliferation was seen ($p=0.02$ for low concentration and $p=0.03$ for high concentration; paired t-test) (Figure 2.39). Treatment of PANC-1 cells with combined S100A8-GST and S100A9-GST proteins produced a

significant increase in proliferation at both low and high concentrations (p=0.02 for low and p=0.03 for high concentration; paired t-test). At 24h only low concentrations (0.4μg/mL) of S100A8-GST and S100A9-GST managed to induce significant growth in PANC-1 cells. At 36h no significant growth induction by the recombinant proteins was measured compared to the GST controls.

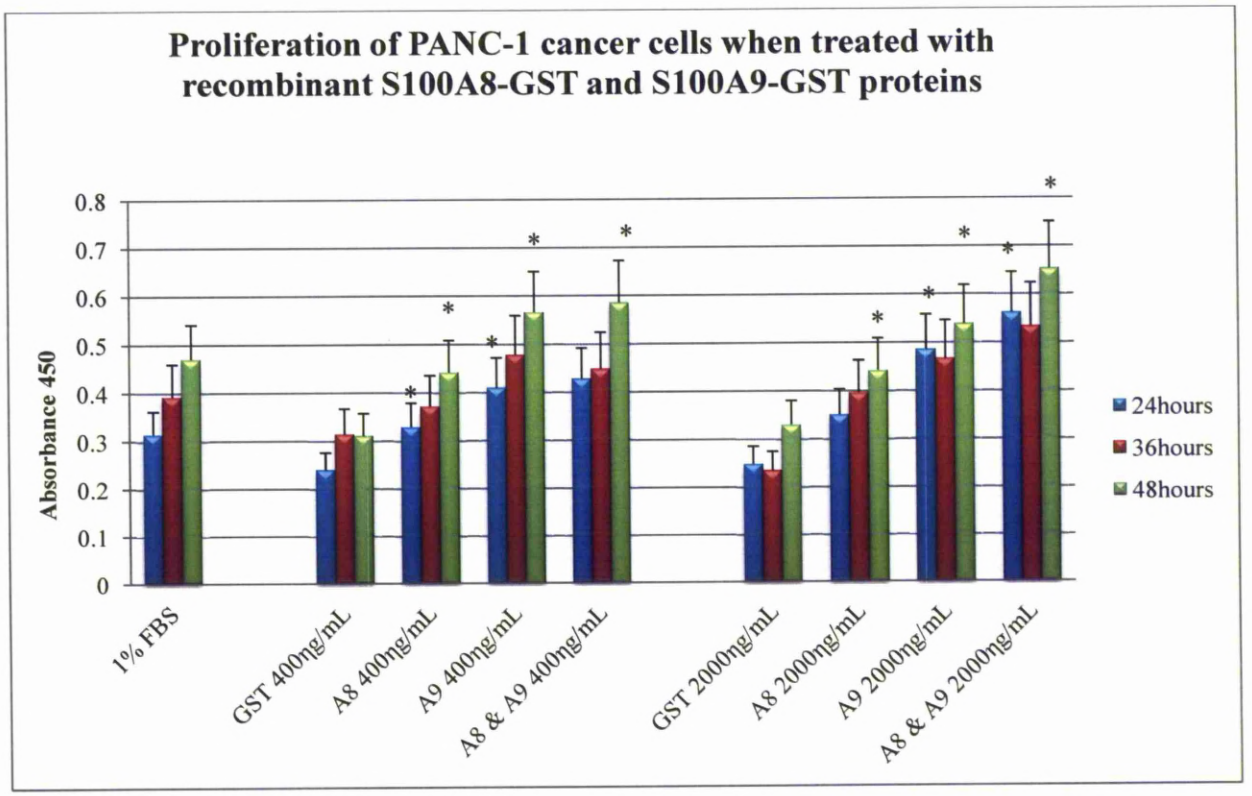


Figure 2.39: MTT proliferation assay for PANC-1 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$)

SUIT-2 cells showed a significant increase in proliferation compared to respective GST control after 36h of incubation (n=3, conducted in quintet) (Figure 2.40), when treated with a high concentration (2μg/mL) of S100A8-GST (p=0.01; paired t-test), S100A9-GST (p=0.05; paired t-test) and combined S100A8-GST and S100A9-GST (p= 0.02; paired t-test) recombinant proteins. The response of SUIT-2 cancer cell lines to low concentration f proteins did not exhibit a significant increase in growth.

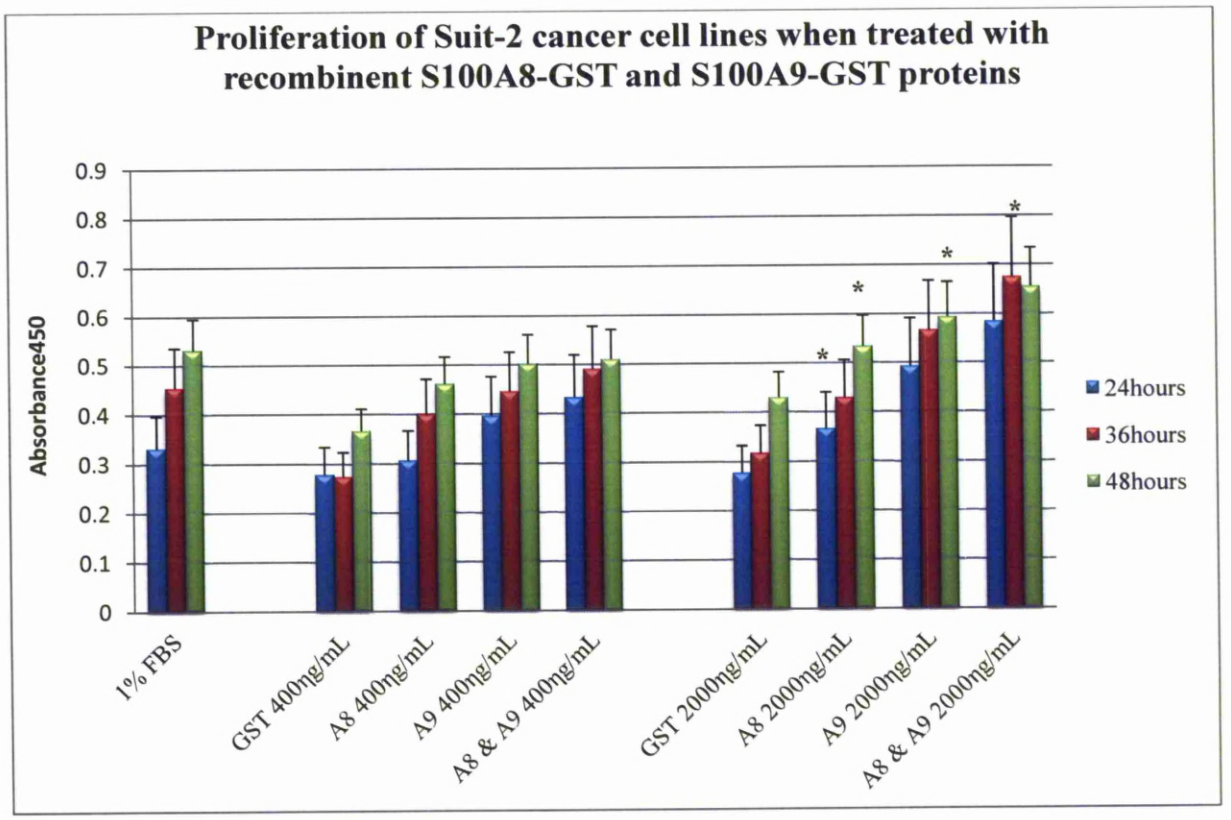


Figure 2.40: MTT proliferation assay for SUIT-2 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$)

MIA PaCa-2 cells demonstrated a significant increase in growth at 48 hours with high concentrations of S100A8-GST ($p=0.01$; paired t-test), S100A9-GST ($p=0.05$; paired t-test) and combined S100A8-GST and S100A9-GST ($p=0.04$; paired t-test) recombinant proteins (n=3, experiments conducted in quintet) (Figure 2.41). After 24h and 36h of treatment with S100 proteins, MIA PaCa cancer cells showed an increase in proliferation however, this did not attain statistical significance.

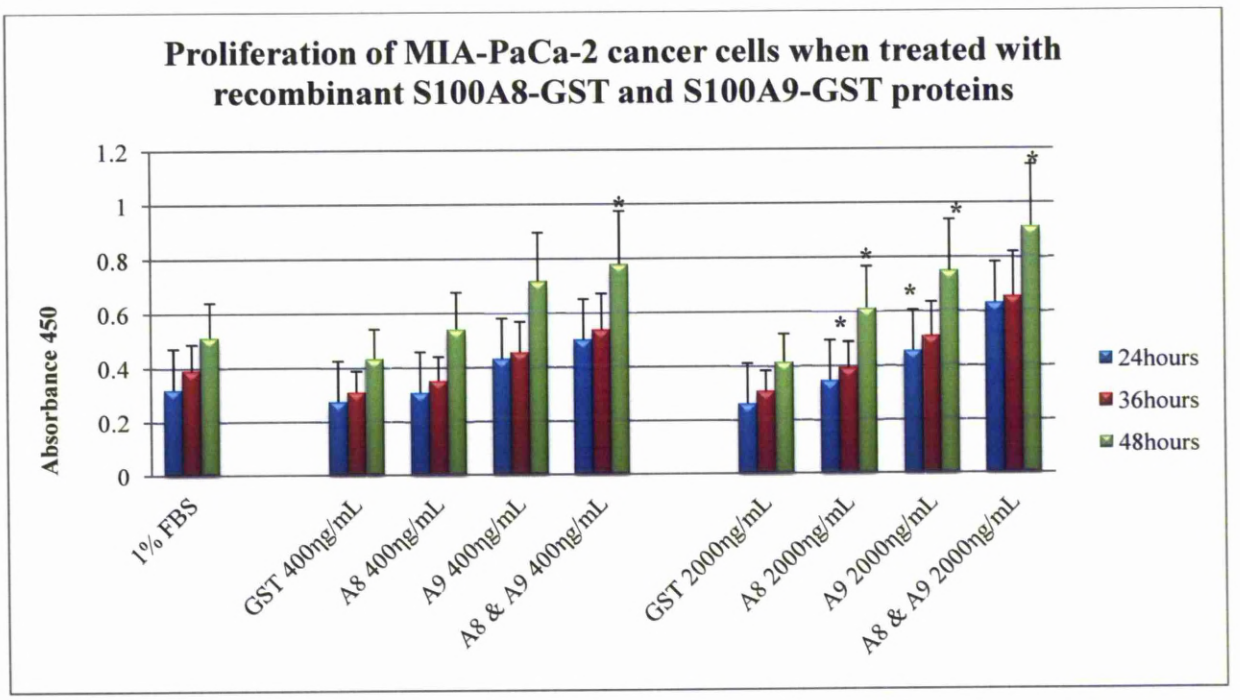


Figure 2.41: MTT proliferation assay for MIA PaCa-2 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$)

The effects of S100A8-GST and S100A9-GST on Colorectal cancer cell proliferation

Recombinant S100A8-GST and S100A9-GST proteins induced proliferation of the colorectal cancer cell lines SW-837 and SW-480 alike. At concentrations of 0.4μg/mL, neither S100A8-GST nor S100A9-GST significantly increased the proliferation of **SW837** cells (n=2, experiments conducted in quintet) (Figure 2.42) relative to GST, however combined S100A8-GST and S100A9-GST protein was seen to induce a significant degree of proliferation at 24h and 36h respectively ($p=0.03$ and $p=0.009$ respectively; paired t-test). When applied at 2μg/mL a significant increase in the proliferation of SW-837 cells was observed when the proteins were used singly or in combination after 24h, 36h and 48 hours of incubation (S100A8: $p < 0.01$, S100A9-GST: $p< 0.01$, S100A8-GST& S100A9-GST: $p<0.01$).

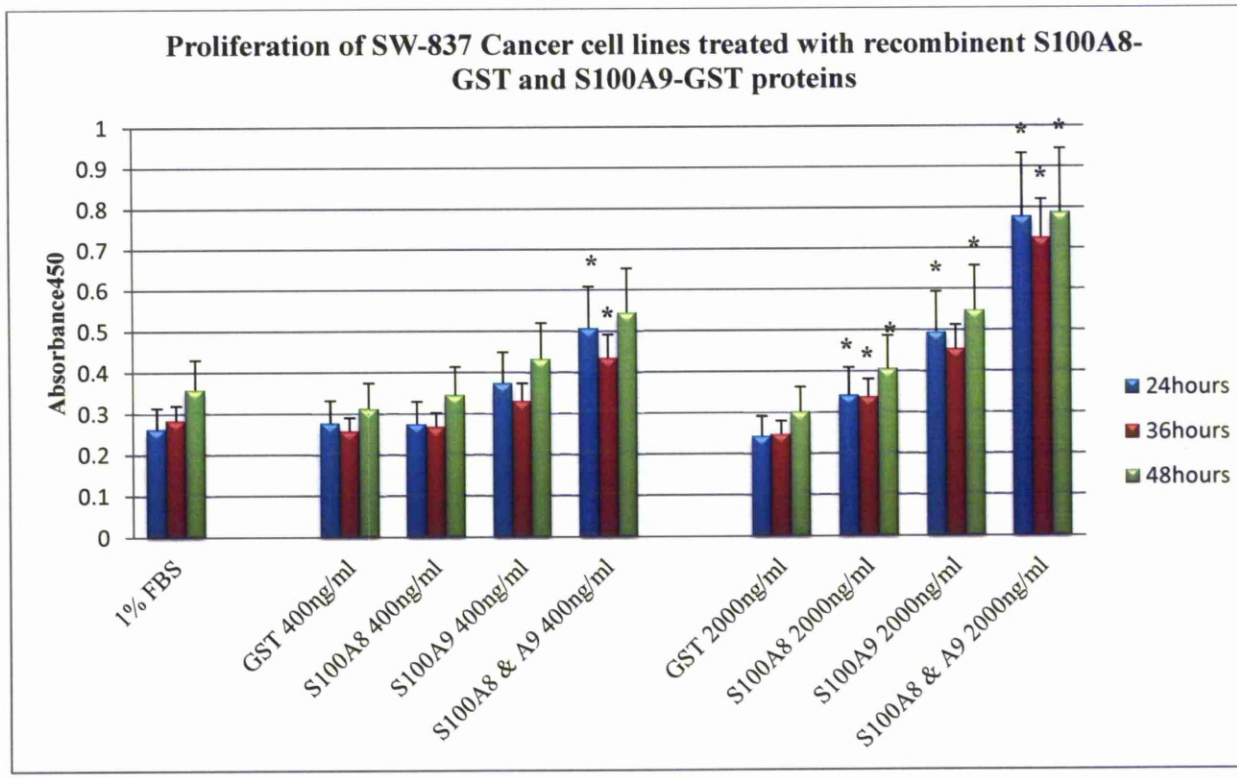


Figure 2.41: MTT proliferation assay for SW-837 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

The proliferative response of **SW-480** (n=3, experiments conducted in quintet) (Figure 2.42) cells did not show a significant increase in growth with S100A8-GST at low concentration (0.4µg/mL) however did show an increased proliferative response to S100A9-GST at 36h and 48h ($p= 0.05$ and $p=0.01$ respectively paired t-test). A significant increase however, in proliferation was observed with S100A8-GST (2µg/mL) at 24 and 36 hours ($p<0.01$; paired t-test). Similarly S100A9-GST at higher

concentrations (2μg/mL) demonstrated a significant increase in proliferation at 24h and 36h but not at 48h (p= 0.01 and p=0.05 respectively; paired t-test). Combined S100A8-GST and S100A9-GST at both 0.4μg/mL and 2μg/mL produced a significant increase in proliferation of SW-480 at 24h and 36h (at 0.4μg/mL: p=0.05 and p=0.02 respectively; at 2μg/mL: p=0.04 and p=0.04 respectively; paired t-test).

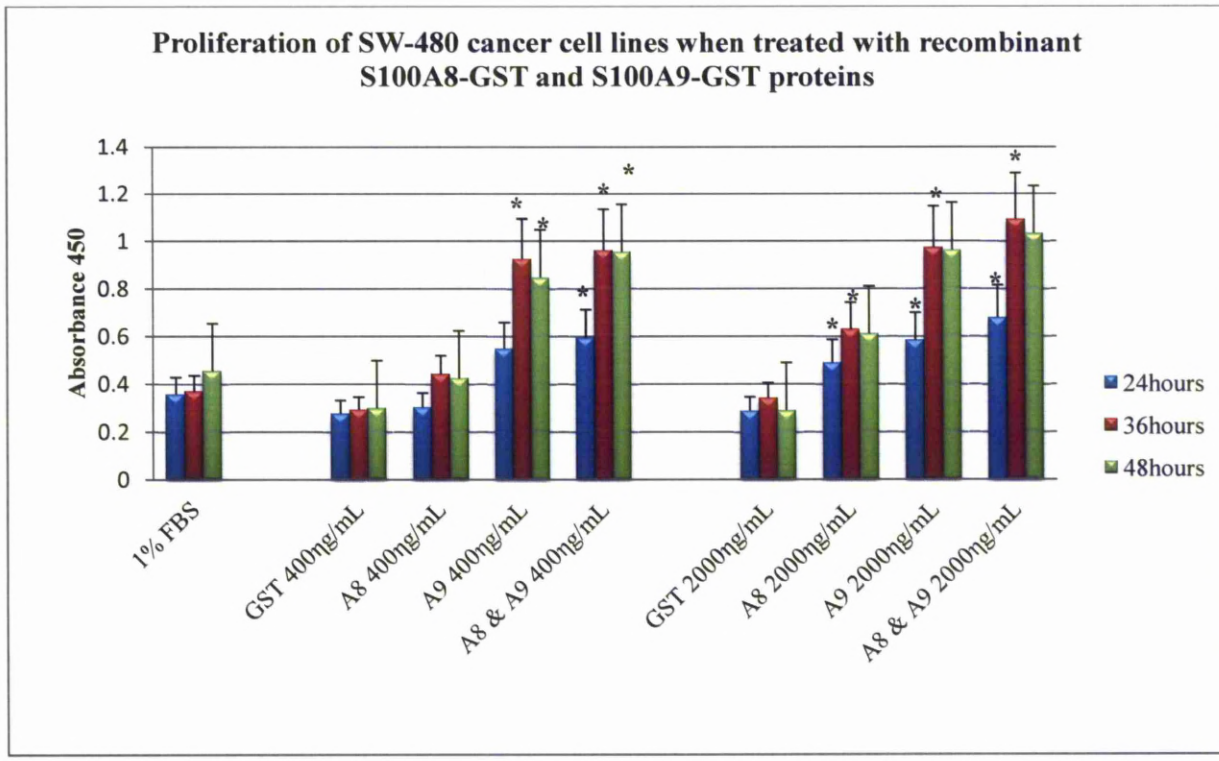


Figure 2.42: MTT proliferation assay for SW480 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

The effects of S100A8-GST and S100A9-GST on SMAD4 positive and negative colorectal clones proliferation

Differential response of S100A8-GST and S100A9-GST recombinant proteins in stable SMAD4 re-expressing clones of colorectal cancer cell lines of SW480 was also observed. The varied expression of SMAD4 in these cell lines has been shown in the motility section previously.

The SMAD4 negative subclone **SWK3** (n=3, experiments conducted in quintet) (Figure 2.43) demonstrated a significant increase in proliferation compared to GST controls when treated with high concentration (2μg/mL) of S100A9-GST recombinant protein only ($p=0.05$ paired t-test at 48 hours). This was not the case with S100A8-GST protein at both low and high concentration. Treatment with low concentration S100A9-GST on its own or combination with S100A8-GST was not undertaken for this cell line. Treatment with combined S100A8-GST and S100A9-GST at 2μg/mL induced a significant proliferative response at 48h ($p= 0.05$; paired t-test) with the response being equal to that of S100A9-GST alone.

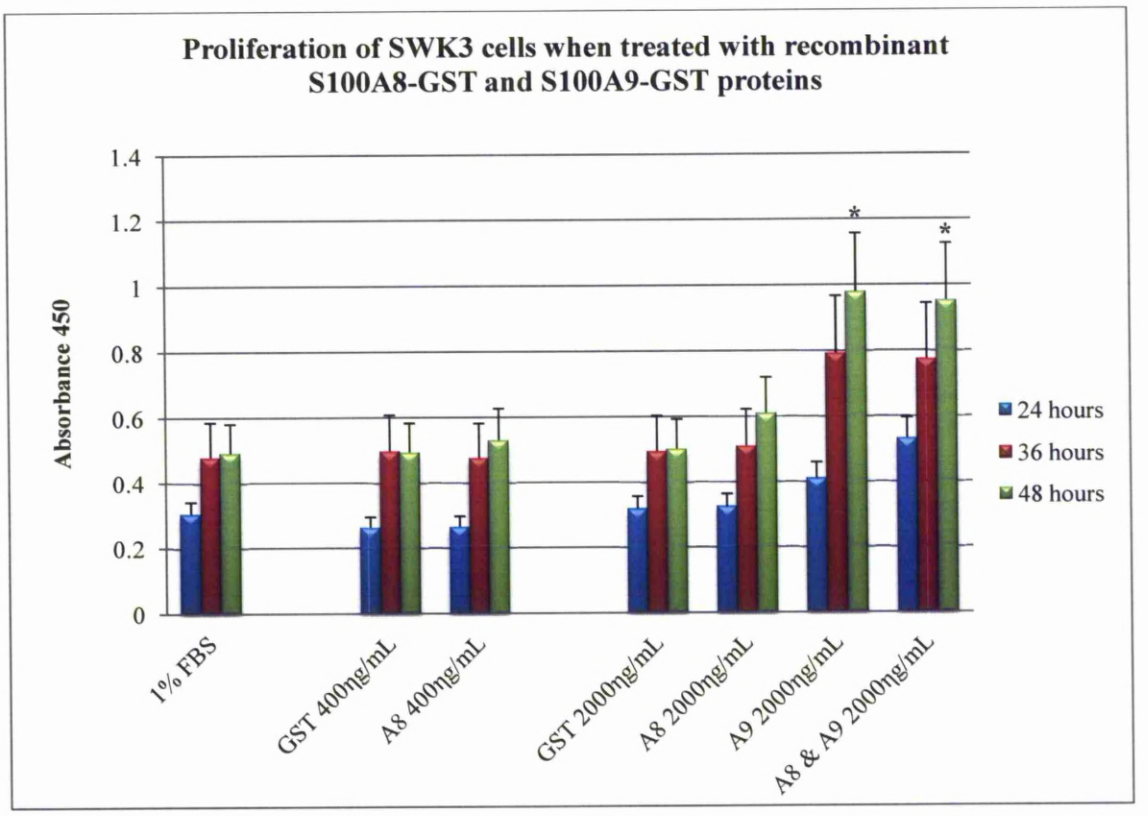


Figure 2.43: MTT proliferation assay for SWK3 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

Interesting the **SWD1** (Figure 2.44) (n=2 experiments conducted in quintet) and **SWD14** (Figure 2.45) (n=2 experiments conducted in quintet) subclones did not exhibit an increase in proliferative response to low (0.4µg/mL) concentration of S100A8-GST, and in the case of SWD14 to S100A9-GST proteins. At a higher concentration (2µg/mL) S100A8-GST did not attain a statistically significant increase in proliferation for both cell lines either. Treatment with high concentration of S100A9-GST on its own or combination with S100A8-GST induced a significant increase in proliferation in both cell

lines at 48h (SWD1 p=0.03; SWD14 p=0.006; paired t-test for S100A9-GST) and (SWD1 p=0.01; SWD14 p=0.05; paired t-test for S100A8-GST & S100A9-GST).

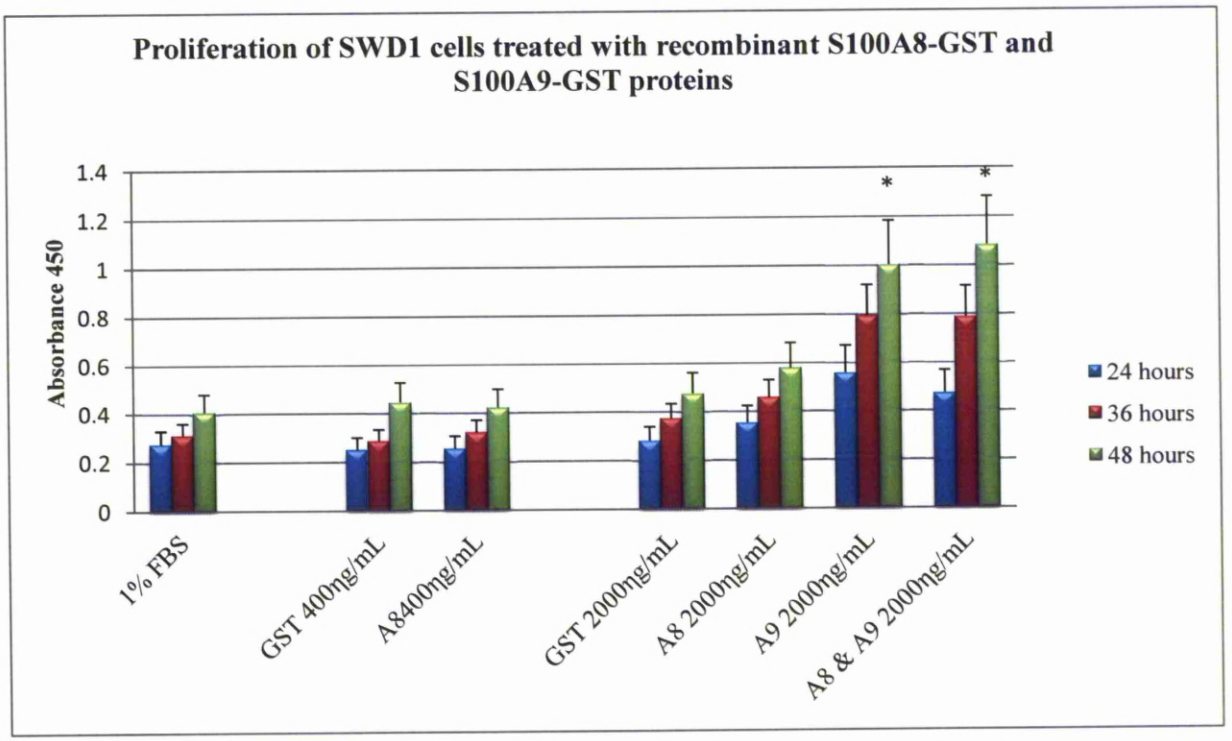


Figure 2.44: MTT proliferation assay for SWD1 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

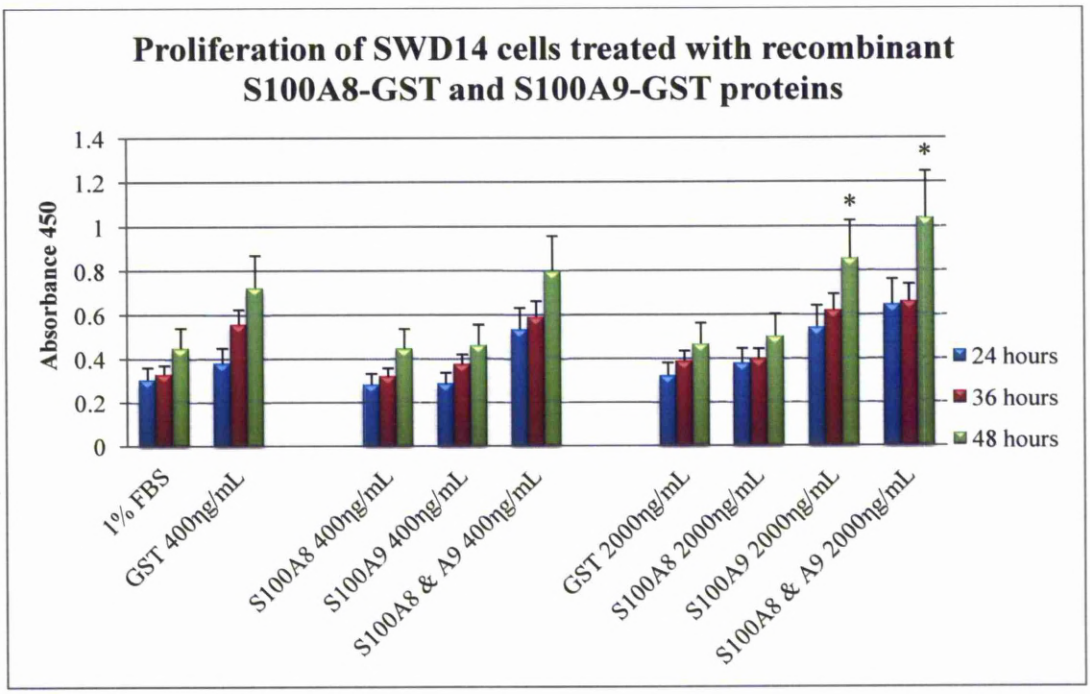


Figure 2.45: MTT proliferation assay for SWD14 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

The subclone **SWD20** (n=3 experiments conducted in quintet) (Figure 2.46) which expressed the highest quantity of SMAD4, showed a positive proliferative response to recombinant S100A9-GST at both low and high concentration at 36h and 48h ($p=0.01$ and $p=0.03$ respectively for 0.4μg/mL) ($p=0.002$ and $p=0.05$ respectively for (2μg/mL). S100A8-GST induced a significant increase in proliferation at 2μg/mL at 48h ($p= 0.05$ paired t-test). The combination of both proteins resulted in a significant increase in proliferation at both low (0.4μg/mL) and high concentration (2μg/mL) at 48 hours respectively ($p<0.001$ and $p=0.04$ respectively; paired t-test).

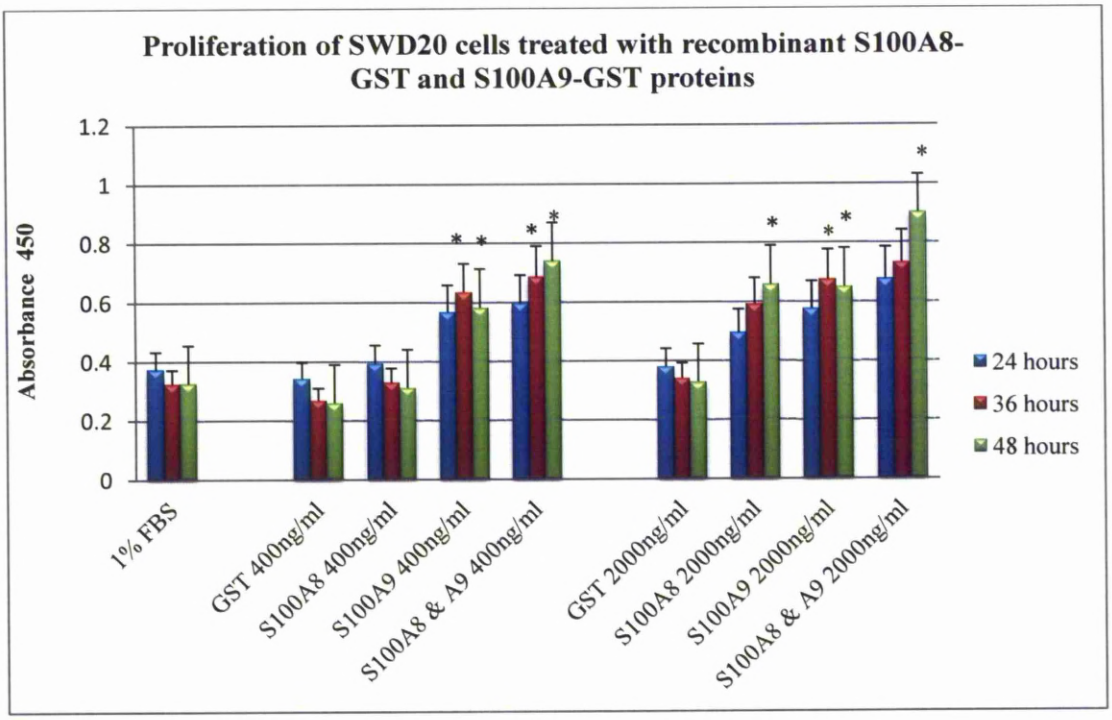


Figure 2.46: MTT proliferation assay for SWD20 cells following 24h, 36h and 48h of incubation with recombinant S100A8-GST (denoted A8) or S100A9-GST (denoted A9) measuring the absolute absorbance. * denotes a significant result ($p<0.05$).

11.6 Discussion

The characterisation of S100A8- and S100A9-positive myeloid cells in the pancreatic cancer microenvironment as previously mentioned revealed a significant association between the numbers of S100A8/S100A9 expressing cells and the SMAD4 status of the tumour (Sheikh et al., 2007). Moreover, in vitro experimentation using conditioned media from pancreatic cancer cell lines induced expression of these proteins in monocytic cell line HL-60. I was however not able to establish a conclusive differential protein expression pattern of S100A8 and S100A9 in HL-60 cells based on the SMAD4 status of the cancer cells from which the conditioned media was derived. The inflammatory chemoattractants S100A8 and S100A9, apart from serving as biomarkers of inflammatory disease conditions are now recognised to play an important role in the pathogenesis and progression of cancer (Ghavami et al., 2009).

Using a mouse model of colon cancer, Kitamura *et al* (Kitamura et al., 2007) reported that SMAD4-negative tumours recruited a specific type of myeloid cell, which promoted invasion through crosstalk with tumour cells. As previously mentioned Hiratsuka *et al.* showed that S100A8/A9 were powerful chemoattractants whose tumour induced presence in the lungs of tumour-bearing mice could stimulate the migration of Lewis Lung carcinoma cells and B16 melanoma cells to that organ. The ability of conditioned media from pancreatic cancer to induce S100A8 and S100A9 also indicated crosstalk channels existing in the pancreatic microenvironment. Therefore, I sought to determine the response of tumour cells to S100A8 and S100A9 in terms of their motility and

proliferation. In this process, I also aimed to understand whether the SMAD4 status of the cancer affected growth or motility of the tumours when treated with recombinant S100A8 and S100A9 proteins.

Both recombinant S100A8 and S100A9 proved to be highly chemotactic and proliferative for pancreatic cancer cell lines, regardless of whether the cells expressed SMAD4 or not. The pancreatic cancer cells showed a response in terms of growth and proliferation compared to the controls in all three cell lines with statistical significance being attained.

The colorectal cell lines SW-480 and SW-837 both underwent proliferation and chemotaxis in response to recombinant S100A8 and S100A9 proteins. Neomycin-resistant clonal derivatives of SW-480 cells, stably re-expressing SMAD4 (SWD20) at high levels and low level expressing transfectants (SWK3, SWD14, SWD1) all showed enhanced motility in response to S100A8 and S100A9 despite differing levels of SMAD4. Moreover, these cells all demonstrated significant cheomotaxis, the proliferation response of the SMAD4 clones SWD1, SWD14 and SWK3 was poor. Transient SMAD4 knockdown in PANC-1 was accompanied by an overall decrease in baseline motility however, compared to controls both proteins exerted chemotactic effects on the cells. Experiments using transient SMAD4 knockdown cells were only undertaken twice and therefore no substantial conclusions could be drawn. The possible mechanism by which these proteins exert their effects has been reported by Ghavami *et al.* (Ghavami et al., 2008b) who demonstrated S100A8 and S100A9 proteins activated the multiligand receptor, RAGE, triggering the mitogen-activated protein kinase signalling pathway.

My colleague Mr. Chin Ang who not only helped me with some aspects of this project took over the project after I left the department and conducted a series of further experiments to elucidate the effects of SMAD4 status had on S100A8-GST and S100A9-GST induced migration activity (Ang et al., 2010). He revisited the transient siRNA-mediated depletion of SMAD4 in PANC-1 cells and also produced a transient knockdown of SWD20 (SMAD4 expressing subclone of SW-480) cells in a similar manner as described above. He successfully demonstrated that knockdown of SMAD4 in both cell lines resulted in an approximate 50% decrease in basal migration compared to respective controls, which was consistent with previous findings (Ang et al., 2010). More importantly, he showed a significant reduction in motility and a loss of responsiveness to S100A8-GST, but not S100A9-GST when cells underwent a transient knockdown of SMAD4. He also demonstrated that SMAD4 re-expressing SW-480 subclones have a differential response to S100A8 and S100A9 proteins based on their levels of SMAD4 expression. Interestingly, while the SMAD4-restored SWD20 cells showed increased proliferation in response to S100A8-GST and S100A9-GST, their SMAD4-negative clonal counterpart, SWK3 cells showed increased proliferation in response to S100A9-GST only. Both of these experiments suggested that SMAD4 was important in regulating the proliferative response to exogenous S100A8 (Ang et al., 2010). I was unable to produce similar results with the knockdown experiments as by Mr. Ang as primarily I undertook them towards the end of my research period and additionally these experiments were still in the phase of optimisation.

It can be postulated that myeloid cells (monocytes) expressing both S100A8 and S100A9 are recruited to the tumour microenvironment in response to growth factors which are

produced by the tumours cells and other stromal components alike. The production of S100A8/A9 may promote further recruitment of inflammatory cells as well as aiding cancer cell growth and invasion. A relationship between the phenotype of the tumour cells i.e. their SMAD4 status and S100A8 and S100A9 does exist as demonstrated by Ang *et al.* (Ang et al., 2010). His work indicates that the crosstalk between SMAD4-negative cancer cells and myeloid cells occurs in a microenvironment that involves S100A9 to a greater extent than S100A8 and where the cancer cells respond better to S100A9 than S100A8. Either way experiments undertaken by Ang *et al.* and myself indicate that crosstalk between the cancer cells and myeloid cells does occur with S100A8 and S100A9 being 2 proteins at the crossroads.

PART FOUR: CONCLUSIONS AND REFERENCES

CHAPTER 12

CONCLUSIONS

Pancreatic cancer is one of the most fatal solid organ malignancies, characterised by a dense desmoplastic stroma accounting for the bulky fibrotic nature of the tumour. Despite advances in surgical techniques and oncology, outcomes remain poor. Although a detailed understanding of the signature molecular events which occur in pancreatic cancer exists, the mechanisms linking these genetic changes to the aggressive nature of this disease remains a field of active research.

Recent years have seen the focus shift from the tumour cells as direct drivers of carcinogenesis to the wider tumour microenvironment. This complex ecosystem is composed of tumour cells, resident and infiltrating non-tumour cells (termed the 'stroma') along with chemomediators (Joyce and Pollard, 2009; Marchesi et al., 2012; Neesse et al., 2011; Shields et al., 2012b; Vincent et al., 2011). A distinct molecular 'crosstalk' between the host and its surrounding cells is the subject of study in a variety of cancer settings and plays an essential role in carcinogenesis, angiogenesis, invasion/metastasis and evasion of the host immune systems. Locally activated cellular and extracellular elements of the host microenvironment secrete molecules that can influence the malignant phenotype modifying the proliferative and invasive behaviour of the tumour, however these interactions are often than not complex and poorly understood.

Cells of myeloid linage are one such component of the dense stroma with monocytes and macrophages having a major role in all aspects of tumour biology and invasion, making them essential regulators in carcinogenesis (Coffelt et al., 2009; Ruffell et al., 2012) (Joyce and Pollard, 2009). S100A8 and S100A9, besides being upregulated in cancer

cells have extensively been reported to be expressed in immature monocytes and cells of the myeloid lineage. Their role in tumour progression as regulators of the "pre-metastatic niche" has certainly been a breakthrough in better understanding of their function in carcinogenesis (Srikrishna, 2012). Moreover, these proteins have also been shown to carry a prognostic significance in a variety of cancers aiding cell motility through the multiligand receptor, RAGE signalling pathway.

My experimental work followed on from the discovery of S100A8 and S100A9 proteins in the stromal elements of microdissected pancreatic cancer samples using 2DE proteomic analysis. I was able to further validate this, demonstrating that ductal elements of pancreatic cancer did not express S100A8 and S100A9 proteins and it was stromal myeloid cells, that is stromal monocytes that exhibited high levels of expression of these proteins. Additionally, none of the other stromal cells demonstrated any expression of these proteins as seen in the co-immunoflorescence experiments. Immunohistochemical analysis of the TMA indicated varying numbers of cells expressing these proteins per tumour core with SMAD4 negative tumours having significantly fewer number of S100A8-postive cells in their stroma compared to S100A9-positive cells. Moreover, the ratio of S100A9/S100A8 positive cells was significantly higher in SMAD4 negative tumours compared to SMAD4 positive tumours (Sheikh et al., 2007). Thus, a strongly negative relationship between the expression of SMAD4 in tumours cells and the expression of S100A8 in stromal monocytes was established. This indicated channels of

crosstalk between the pancreatic cancer cells and the stroma which was potentially influenced by the SMAD4 status of the tumour.

The presence of S100A8 in a subset of S100A9-positive pancreatic cancer stromal cells on immunoflorescence mirrored findings published by Zwadlo *et al.* that the presence of both S100 proteins in monocytes is expressed at early stages of monocyte differentiation being down regulated during maturation to macrophages (Zwadlo et al., 1988). The varying number of cells per tumour core expressing S100A8 and S100A9 indicated that the tumour cells may well be influencing this process with SMAD4 being a key regulator. Levels of expression of both of these proteins did not influence survival and no association to patient parameters was established other than their relation to the tumour phenotype.

In vitro interaction experiments between pancreatic cancer cell secretome and monocytes demonstrated induction of S100A8 and S100A9 proteins in both primary monocytes and monocytic cell lines indicating crosstalk between both cell types. The presence of VEGF-A and TGF-β soluble factors in the tumour secretome, suggested their potential role in inducing S100A8 and S100A9 expression as is the case in other cancer models in the literature. Immunohistochemical analysis of the pancreatic cancer TMA also demonstrated a strong presence of TGF-β1 in both the tumour and stromal compartments. Importantly, only the presence of tumour nuclear TGF-β1 expression significantly

correlated to stromal S100A8 and S100A9 expression with no correlation seen between the S100 proteins and stromal TGF-β1 expression. This suggested that the presence of TGF-β1 in the cancer cells was one of the mediators effecting the expression of S100A8 and S100A9 in monocytes both in cellular experiments and in the microenvironment. TGF-β1 overexpression was also seen to be associated with a poor 2-year survival that is consistent with the literature. In the face of TGF-β1 having no correlation to patient parameters i.e. perineural invasion, lymphovascular invasion and metastasis this is a significant finding warranting further validation in a larger cohort of patients. It can be concluded from my immunohistochemical and cellular experiments that monocytes expressing both S100A8 and S100A9 are recruited to the tumour microenvironment in response to growth factors such as TGF-β1 produced by the tumours cells. Once recruited from the blood stream they initially express both S100A8 and S100A9 and as they mature, they lose S100A8 expression, leaving only S100A9 which is also subsequently lost as the cells matures further to form macrophages. The presence of CD68 macrophages and the strong correlation they have to the number of S100A8- and S100A9-positive cells in the cancer stroma further consolidates this process and the role cells of myeloid linage have in the cancer microenvironment. I was also able to postulate that the rate of differentiation of monocytes once recruited to the microenvironment could well be influenced by the tumour SMAD4 expression (Sheikh *et al.*, 2007). From my IHC data of the TMA it can be postulated that in SMAD4-negative tumours S100A8 expression becomes very transient, leaving relatively greater numbers of cells expressing only S100A9; thought my experimental data did not support these findings, data from Ang *et al.* did support this hypothesis (Ang *et al.*, 2010). Likewise other tumour

characteristics may influence the rate of maturation and expression of S100A8 and S100A9 in the pancreatic cancer microenvironment, these have yet to be evaluated.

The induction of S100A8 and S100A9 in monocytes from pancreatic cancer cell conditioned medium and its associations to the cancer phenotype on the IHC analysis indicated a crosstalk channel. Therefore, I sought to determine the response recombinant S100A8 and S100A9 proteins had on cancer cell motility and proliferation. In this process, I also aimed to understand whether the SMAD4 status of the cancer affected growth or motility of the tumours when treated with recombinant S100A8 and S100A9 proteins. It can be concluded that both proteins proved to be highly chemotactic and proliferative for pancreatic and colorectal cell lines cancer cell lines, regardless of whether the cells expressed SMAD4 or not. Experiments with neomycin-resistant clonal derivatives of SW-480 cells, stably re-expressing SMAD4 and transient knockdown SMAD4 cellular experiments, all showed enhanced motility in response to S100A8 and S100A9 despite differing levels of SMAD4. I am unable to draw any substantial conclusions as to whether the SMAD4 status of the cancer cell effects its chemotactic or proliferative response to the S100 proteins.

Future direction of work should me aimed at elucidating the effects SMAD4 status has on S100A8-GST and S100A9-GST induced migration activity. My colleague Mr C. Ang revisited the transient siRNA-mediated depletion of SMAD4 in PANC-1 cells

experiments. He successfully demonstrated that knockdown of SMAD4 produced a significant reduction in motility and a loss of responsiveness to S100A8-GST, but not S100A9-GST when cells underwent a transient knockdown of SMAD4. He also demonstrated that SMAD4 re-expressing SW-480 subclones have a differential response to S100A8 and S100A9 proteins based on their levels of SMAD4 expression. SMAD4-restored SWD20 cells showed increased proliferation in response to S100A8-GST and S100A9-GST, with their SMAD4-negative clonal counterpart, SWK3 cells showing increased proliferation in response to S100A9-GST only. Both of these experiments suggested that SMAD4 was important in regulating the proliferative response to exogenous S100A8 (Ang et al., 2010).

It can be concluded that monocytes expressing both S100A8 and S100A9 are recruited to the tumour microenvironment in response to growth factors such as TGF-β which are produced by the tumours cells and other stromal components. The production of S100A8/A9 may promote further recruitment of inflammatory cells but is influential in aiding cancer cell growth and invasion. A relationship between the phenotype of the tumour cells i.e. their SMAD4 status and S100A8 and S100A9 does exist as demonstrated by Ang *et al.* (Ang et al., 2010). His work indicates that the crosstalk between SMAD4-negative cancer cells and myeloid cells occurs in a microenvironment that involves S100A9 to a greater extent than S100A8 and where the cancer cells respond better to S100A9 than S100A8. Either way experiments undertaken by Ang *et al.* and myself indicate that crosstalk between the cancer cells and myeloid cells does occur with S100A8 and S100A9 being 2 proteins at the crossroads.

This understanding of the relationship between cancer cells and S100A8/S100A9 proteins which is just one of the molecular interactions in the tumour microenvironment representing a small cog in a large machine. Certainly further studies to evaluate the mechanisms of S100A8/A9 signalling in the pancreatic and colorectal cancer tumour microenvironment will shed light on how these proteins influence the processes of tumour development and spread. Further cancer mouse model experiments using blocking antibodies to these S100 proteins will provide the first steps for utilisation of these proteins as therapeutic targets.

CHAPTER 13

REFERENCES

- Aarnio, M., Mecklin, J. P., Aaltonen, L. A., Nystrom-Lahti, M., and Jarvinen, H. J. (1995). Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Int J Cancer *64*, 430-433.
- Agbunag C, and Bar-Sagi D (2004). Oncogenic K-ras drives cell cycle progression and phenotypic conversion of primary pancreatic ductal cells. Canc Res *64*, 5659-5663.
- Albazaz, R., Verbeke, C. S., Rahman, S. H., and McMahon, M. J. (2005). Cyclooxygenase-2 expression associated with severity of PanIN lesions: a possible link between chronic pancreatitis and pancreatic cancer. Pancreatology *5*, 361-369.
- Alexakis, N., Halloran, C., Raraty, M., Ghaneh, P., Sutton, R., and Neoptolemos, J. P. (2004). Current standards of surgery for pancreatic cancer. Br J Surg *91*, 1410-1427.
- Alexander CM, and Werb Z (1992). Targeted disruption of the tissue inhibitor of metalloproteinase gene increases the invasive behaviour of primitive mesenchymal cells derived from embyonic stem cells in vitro. J Cell Biol *118*, 727-739.
- Alguacil, J., and Silverman, D. T. (2004). Smokeless and other noncigarette tobacco use and pancreatic cancer: a case-control study based on direct interviews. Cancer Epidemiol Biomarkers Prev *13*, 55-58.
- Altomare, D. A., Tanno, S., De Rienzo, A., Klein-Szanto, A. J., Tanno, S., Skele, K. L., Hoffman, J. P., and Testa, J. R. (2003). Frequent activation of AKT2 kinase in human pancreatic carcinomas. J Cell Biochem *88*, 470-476.
- Andea, A., Sarkar, F., and Adsay, V. N. (2003). Clinicopathological correlates of pancreatic intraepithelial neoplasia: a comparative analysis of 82 cases with and 152 cases without pancreatic ductal adenocarcinoma. Mod Pathol *16*, 996-1006.
- Andoh, A., Takaya, H., Saotome, T., Shimada, M., Hata, K., Araki, Y., Nakamura, F., Shintani, Y., Fujiyama, Y., and Bamba, T. (2000). Cytokine regulation of chemokine (IL-8, MCP-1, and RANTES) gene expression in human pancreatic periacinar myofibroblasts. Gastroenterology *119*, 211-219.
- Andreasen PA, Kjoller L, Christensen L, and Duffy M (1997). The urokinase-type palsminogen activator system in cancer metastasis: a review. Int J Cancer *72*, 1-22.
- Andreotti, G., and Silverman, D. T. (2012). Occupational risk factors and pancreatic cancer: a review of recent findings. Mol Carcinog *51*, 98-108.
- Ang, C. W., Nedjadi, T., Sheikh, A. A., Tweedle, E. M., Tonack, S., Honap, S., Jenkins, R. E., Park, B. K., Schwarte-Waldhoff, I., Khattak, I., *et al.* (2010). Smad4 loss is associated with fewer S100A8-positive monocytes in colorectal tumors and attenuated response to S100A8 in colorectal and pancreatic cancer cells. Carcinogenesis *31*, 1541-1551.
- Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B., and De Larco, J. E. (1983). Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci U S A *80*, 6264-6268.
- Arao S, Masumoto A, and Otsuki M (2000). Beta1 integrins play an essential role in r and invasion of pancreatic carcinoma cells. Pancreas *20*, 129-137.

- Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circ Res *97*, 512-523.
- Bachem MG, Schneider E, Groß H, Weidenbach H, Schmid R, Menke A, Siech M, Beger HG, Grünert A, and Adler G (1998). Identification, culture, and characterisation of pancreatic stellate cells in rats and humans. Gastroenterology *115*, 421-432.
- Bachem, M. G., Schunemann, M., Ramadani, M., Siech, M., Beger, H., Buck, A., Zhou, S., Schmid-Kotsas, A., and Adler, G. (2005). Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. Gastroenterology *128*, 907-921.
- Baj-Krzyworzeka, M., Baran, J., Szatanek, R., Stankiewicz, D., Siedlar, M., and Zembala, M. (2004). Prevention and reversal of tumor cell-induced monocyte deactivation by cytokines, purified protein derivative (PPD), and anti-IL-10 antibody. Cancer Immun *4*, 8.
- Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.
- Balkwill, F. (2004). Cancer and the chemokine network. Nat Rev Cancer *4*, 540-550.
- Baran, B., Bechyne, I., Siedlar, M., Szpak, K., Mytar, B., Sroka, J., Laczna, E., Madeja, Z., Zembala, M., and Czyz, J. (2009). Blood monocytes stimulate migration of human pancreatic carcinoma cells in vitro: the role of tumour necrosis factor - alpha. Eur J Cell Biol *88*, 743-752.
- Bardeesy, N., Aguirre, A. J., Chu, G. C., Cheng, K. H., Lopez, L. V., Hezel, A. F., Feng, B., Brennan, C., Weissleder, R., Mahmood, U., *et al.* (2006). Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proc Natl Acad Sci U S A *103*, 5947-5952.
- Bardeesy, N., and DePinho, R. A. (2002). Pancreatic cancer biology and genetics. Nat Rev Cancer *2*, 897-909.
- Barger SW, Wolchok SR, and Van Eldik LJ (1992). Disulfide linked S100 beta dimers and signal transduction. Biochim Biophys Acta *1160*, 105-112.
- Bartsch DK, Sina-Frey M, Lang S, and et al. (2002). CDKN2A germline mutations in familial pancreatic cancer. Ann Surg *236*, 730-737.
- Basso D, Valerio A, Seraglia R, Mazza S, Piva MG, Greco E, Fogar P, Gallo N, Pedrazozoli S, Tiengo A, and Plebani M (2002). Putatve pancreatic cancer associated diabetogenic factor: 2030 MW peptide. Pancreas *24*, 8-14.
- Bellone, G., Smirne, C., Mauri, F. A., Tonel, E., Carbone, A., Buffolino, A., Dughera, L., Robecchi, A., Pirisi, M., and Emanuelli, G. (2006). Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. Cancer Immunol Immunother *55*, 684-698.
- Bellone, G., Turletti, A., Artusio, E., Mareschi, K., Carbone, A., Tibaudi, D., Robecchi, A., Emanuelli, G., and Rodeck, U. (1999). Tumor-associated transforming growth factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients. Am J Pathol *155*, 537-547.
- Berger DH (2002). Plasmin/plasminogen system in colorectal cancer. Worl J Surg *26*, 767-771.

- Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, and Hanahan D (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogensis. Nat Cell Biol *2*, 737-744.
- Berntzen, H. B., Munthe, E., and Fagerhol, M. K. (1989). A longitudinal study of the leukocyte protein L1 as an indicator of disease activity in patients with rheumatoid arthritis. J Rheumatol *16*, 1416-1420.
- Berrozpe G, Schaeffer J, Peinado MA, and et al. (1994). Comparative analysis of mutations in the p53 and K-*ras* genes in pancreatic cancer. Int J Cancer *58*, 185-191.
- Besson, A., Dowdy, S. F., and Roberts, J. M. (2008). CDK inhibitors: cell cycle regulators and beyond. Dev Cell *14*, 159-169.
- Bharadwaj, R., and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. Oncogene *23*, 2016-2027.
- Biankin, A. V., Kench, J. G., Colvin, E. K., Segara, D., Scarlett, C. J., Nguyen, N. Q., Chang, D. K., Morey, A. L., Lee, C. S., Pinese, M., *et al.* (2009). Expression of S100A2 calcium-binding protein predicts response to pancreatectomy for pancreatic cancer. Gastroenterology *137*, 558-568, 568 e551-511.
- Biankin, A. V., Morey, A. L., Lee, C. S., Kench, J. G., Biankin, S. A., Hook, H. C., Head, D. R., Hugh, T. B., Sutherland, R. L., and Henshall, S. M. (2002). DPC4/Smad4 expression and outcome in pancreatic ductal adenocarcinoma. J Clin Oncol *20*, 4531-4542.
- Bierie, B., and Moses, H. L. (2006). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer *6*, 506-520.
- Bingle, L., Brown, N. J., and Lewis, C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol *196*, 254-265.
- Bissell, M. J., and Radisky, D. (2001). Putting tumours in context. Nat Rev Cancer *1*, 46-54.
- Bloomston M, Shafi A, Zervos EE, and Rosemurgy AS (2002). TIMP-1 overexpression in pancreatic cancer attenuates tumour growth, decreases implantation and metastasis, and inhibits angiogenesis. J Surg Res *102*, 39-44.
- Bramhall SR, Neoptolemos JP, Stamp G, and Lemione NR (1997). Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs) in human pancreatic carcinoma. J Pathol *182*, 347-355.
- Brentnall, T. A. (2000). Cancer surveillance of patients from familial pancreatic cancer kindreds. Med Clin North Am *84*, 707-718.
- Brown, L. M., Helmke, S. M., Hunsucker, S. W., Netea-Maier, R. T., Chiang, S. A., Heinz, D. E., Shroyer, K. R., Duncan, M. W., and Haugen, B. R. (2006). Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. Mol Carcinog *45*, 613-626.
- Buchholz, M., and Gress, T. M. (2009). Molecular changes in pancreatic cancer. Expert Rev Anticancer Ther *9*, 1487-1497.

- Buchler P, Reber HA, Buchler M, Friess H, Seneza GL, and Hines OJ (2003). Hypoxia inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic carcinoma. Pancreas *26*, 56-64.
- Calhoun, E. S., Jones, J. B., Ashfaq, R., Adsay, V., Baker, S. J., Valentine, V., Hempen, P. M., Hilgers, W., Yeo, C. J., Hruban, R. H., and Kern, S. E. (2003). BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: potential therapeutic targets. Am J Pathol *163*, 1255-1260.
- Calle EE, and Kaaks R (2004). Overweight, obesity and cancer: epidemiological evidence and proposed mechansims. Nat Rev Cancer *4*, 579-588.
- Calle, E. E., Rodriguez, C., Walker-Thurmond, K., and Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med *348*, 1625-1638.
- Campbell, P. J., Yachida, S., Mudie, L. J., Stephens, P. J., Pleasance, E. D., Stebbings, L. A., Morsberger, L. A., Latimer, C., McLaren, S., Lin, M. L., *et al.* (2010). The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature *467*, 1109-1113.
- Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998). Increasing complexity of Ras signaling. Oncogene *17*, 1395-1413.
- Cancer Research, U. (2009). Office for National Statistics, 2009 Cancer Statistics registrations: registrations of cancer diagnosed in 2006, England. http://wwwstatisticsgovuk/statbase/Productasp?vlnk=8843.
- Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.
- Carriere, C., Young, A. L., Gunn, J. R., Longnecker, D. S., and Korc, M. (2011). Acute pancreatitis accelerates initiation and progression to pancreatic cancer in mice expressing oncogenic Kras in the nestin cell lineage. PLoS One *6*, e27725.
- Chandler NM, Canete JJ, and Callery MP (2004). Increased expression of NF-κB subunits in human pancreatic cancer cells. J Surg Res *118*, 9-14.
- Chari, S. T., Leibson, C. L., Rabe, K. G., Timmons, L. J., Ransom, J., de Andrade, M., and Petersen, G. M. (2008). Pancreatic cancer-associated diabetes mellitus: prevalence and temporal association with diagnosis of cancer. Gastroenterology *134*, 95-101.
- Chen, G., Tian, X., Liu, Z., Zhou, S., Schmidt, B., Henne-Bruns, D., Bachem, M., and Kornmann, M. (2010). Inhibition of endogenous SPARC enhances pancreatic cancer cell growth: modulation by FGFR1-III isoform expression. Br J Cancer *102*, 188-195.
- Chen, T. R., Hay, R. J., and Macy, M. L. (1983). Intercellular karyotypic similarity in near-diploid cell lines of human tumor origins. Cancer Genet Cytogenet *10*, 351-362.
- Cheng, P., Corzo, C. A., Luetteke, N., Yu, B., Nagaraj, S., Bui, M. M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., *et al.* (2008). Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. J Exp Med *205*, 2235-2249.
- Chu, G. C., Kimmelman, A. C., Hezel, A. F., and Depinho, R. A. (2007). Stromal biology of pancreatic cancer. J Cell Biochem.

- Chua, Y. J., and Cunningham, D. (2006). Chemotherapy for advanced pancreatic cancer. Best Pract Res Clin Gastroenterol *20*, 327-348.
- Classon M, and Harlow E (2002). The retinoblastoma tumour suppressor in devlopment and cancer. Nat Rev Canc *2*, 910-917.
- Clayton, A., Evans, R. A., Pettit, E., Hallett, M., Williams, J. D., and Steadman, R. (1998). Cellular activation through the ligation of intercellular adhesion molecule-1. J Cell Sci *111 (Pt 4)*, 443-453.
- Coffelt, S. B., Hughes, R., and Lewis, C. E. (2009). Tumor-associated macrophages: effectors of angiogenesis and tumor progression. Biochim Biophys Acta *1796*, 11-18.
- Collins, M. A., Bednar, F., Zhang, Y., Brisset, J. C., Galban, S., Galban, C. J., Rakshit, S., Flannagan, K. S., Adsay, N. V., and Pasca di Magliano, M. (2012). Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. J Clin Invest *122*, 639-653.
- Collisson, E. A., Sadanandam, A., Olson, P., Gibb, W. J., Truitt, M., Gu, S., Cooc, J., Weinkle, J., Kim, G. E., Jakkula, L., *et al.* (2011). Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. Nat Med *17*, 500-503.
- Condeelis, J., and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell *124*, 263-266.
- Corbin, B. D., Seeley, E. H., Raab, A., Feldmann, J., Miller, M. R., Torres, V. J., Anderson, K. L., Dattilo, B. M., Dunman, P. M., Gerads, R., *et al.* (2008). Metal chelation and inhibition of bacterial growth in tissue abscesses. Science *319*, 962-965.
- Costantino, C. L., Witkiewicz, A. K., Kuwano, Y., Cozzitorto, J. A., Kennedy, E. P., Dasgupta, A., Keen, J. C., Yeo, C. J., Gorospe, M., and Brody, J. R. (2009). The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase. Cancer Res *69*, 4567-4572.
- Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell *103*, 481-490.
- Coussens, L. M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.
- Crane, C. H., Varadhachary, G. R., Yordy, J. S., Staerkel, G. A., Javle, M. M., Safran, H., Haque, W., Hobbs, B. D., Krishnan, S., Fleming, J. B., *et al.* (2011). Phase II trial of cetuximab, gemcitabine, and oxaliplatin followed by chemoradiation with cetuximab for locally advanced (T4) pancreatic adenocarcinoma: correlation of Smad4(Dpc4) immunostaining with pattern of disease progression. J Clin Oncol *29*, 3037-3043.
- Crnogorac-Jurcevic, T., Missiaglia, E., Blaveri, E., Gangeswaran, R., Jones, M., Terris, B., Costello, E., Neoptolemos, J. P., and Lemoine, N. R. (2003). Molecular alterations in pancreatic carcinoma: expression profiling shows that dysregulated expression of S100 genes is highly prevalent. J Pathol *201*, 63-74.
- Cross, S. S., Hamdy, F. C., Deloulme, J. C., and Rehman, I. (2005). Expression of S100 proteins in normal human tissues and common cancers using tissue

microarrays: S100A6, S100A8, S100A9 and S100A11 are all overexpressed in common cancers. Histopathology *46*, 256-269.

- Crous-Bou, M., Porta, M., Lopez, T., Jariod, M., Malats, N., Alguacil, J., Morales, E., Fernandez, E., Corominas, J. M., Carrato, A., *et al.* (2007). Lifetime history of tobacco consumption and K-ras mutations in exocrine pancreatic cancer. Pancreas *35*, 135-141.
- Crous-Bou, M., Porta, M., Lopez, T., Jariod, M., Malats, N., Morales, E., Guarner, L., Rifa, J., Carrato, A., and Real, F. X. (2009). Lifetime history of alcohol consumption and K-ras mutations in pancreatic ductal adenocarcinoma. Environ Mol Mutagen *50*, 421-430.
- Cunningham, D., Chau, I., Stocken, D. D., Valle, J. W., Smith, D., Steward, W., Harper, P. G., Dunn, J., Tudur-Smith, C., West, J., *et al.* (2009). Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol *27*, 5513-5518.
- Curran S, and Murray GI (1999). Matrix Metalloproteinases in Tumour Invasion and Metastasis. J Pathol *189*, 300-308.
- Currie, C. J., Poole, C. D., and Gale, E. A. (2009). The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. Diabetologia *52*, 1766-1777.
- Danen EHJ, and Sonnenberg A (2003). Integrins in regulation of tissue development. J Pathol *200*, 471-480.
- Daniel DC (2002). Highlight: BRCA1 and BRCA2 proteins in breast cancer. Microsc Res Tech *59*, 68-83.
- de Braud, F., Cascinu, S., and Gatta, G. (2004). Cancer of pancreas. Crit Rev Oncol Hematol *50*, 147-155.
- De Palma, M., and Naldini, L. (2009). Tie2-expressing monocytes (TEMs): novel targets and vehicles of anticancer therapy? Biochim Biophys Acta *1796*, 5-10.
- De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., Politi, L. S., Sampaolesi, M., and Naldini, L. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell *8*, 211-226.
- De Palma, M., Venneri, M. A., Roca, C., and Naldini, L. (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med *9*, 789-795.
- Degrate, L., Nobili, C., Franciosi, C., Caprotti, R., Brivio, F., Romano, F., Leone, B. E., Trezzi, R., and Uggeri, F. (2009). Interleukin-2 immunotherapy action on innate immunity cells in peripheral blood and tumoral tissue of pancreatic adenocarcinoma patients. Langenbecks Arch Surg *394*, 115-121.
- Detlefsen, S., Sipos, B., Feyerabend, B., and Kloppel, G. (2005). Pancreatic fibrosis associated with age and ductal papillary hyperplasia. Virchows Arch *447*, 800-805.
- Diaz VM, Planaguma J, Thompson TM, Reventos J, and Paciucci R (2002). Tissue plasminogen activator is required for the growth, invasion , and angiogenesis of pancreatic tumour cells. Gastroenetrology *122*, 806-819.
- DiMagno, M. J., and DiMagno, E. P. (2009). Chronic pancreatitis. Curr Opin Gastroenterol *25*, 454-459.

- Dineen, S. P., Lynn, K. D., Holloway, S. E., Miller, A. F., Sullivan, J. P., Shames, D. S., Beck, A. W., Barnett, C. C., Fleming, J. B., and Brekken, R. A. (2008). Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. Cancer Res *68*, 4340-4346.
- Dirkx, A. E., Oude Egbrink, M. G., Wagstaff, J., and Griffioen, A. W. (2006). Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J Leukoc Biol *80*, 1183-1196.
- Donato, R. (2003). Intracellular and extracellular roles of S100 proteins. Microsc Res Tech *60*, 540-551.
- Dowen, S. E., Crnogorac-Jurcevic, T., Gangeswaran, R., Hansen, M., Eloranta, J. J., Bhakta, V., Brentnall, T. A., Luttges, J., Kloppel, G., and Lemoine, N. R. (2005). Expression of S100P and its novel binding partner S100PBPR in early pancreatic cancer. Am J Pathol *166*, 81-92.
- Dranoff G (2004). Cytokines in cancer pathogenesis and cancer therapy. Nat Reviews Cancer *4*, 11-22.
- Duner, S., Lopatko Lindman, J., Ansari, D., Gundewar, C., and Andersson, R. (2010). Pancreatic cancer: the role of pancreatic stellate cells in tumor progression. Pancreatology *10*, 673-681.
- Dvorak HF (2003). How tumours make bad blood vessels and stroma. Am J Pathol *162*, 1747-1757.
- Earl, J., Yan, L., Vitone, L. J., Risk, J., Kemp, S. J., McFaul, C., Neoptolemos, J. P., Greenhalf, W., Kress, R., Sina-Frey, M., *et al.* (2006). Evaluation of the 4q32-34 locus in European familial pancreatic cancer. Cancer Epidemiol Biomarkers Prev *15*, 1948-1955.
- Ebrahimi, B., Tucker, S. L., Li, D., Abbruzzese, J. L., and Kurzrock, R. (2004). Cytokines in pancreatic carcinoma: correlation with phenotypic characteristics and prognosis. Cancer *101*, 2727-2736.
- Egeblad, M., and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*, 161-174.
- Ehrchen, J. M., Sunderkotter, C., Foell, D., Vogl, T., and Roth, J. (2009). The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol.
- El-Hariry I, Pignatelli M, and Lemoine NR (2001). FGF-1 and FGF-2 regulate the expression of E-caherin and catenins in pancreatic adenocarcinoma. Int J Cancer *94*, 652-661.
- El-Serag, H. B., Engels, E. A., Landgren, O., Chiao, E., Henderson, L., Amaratunge, H. C., and Giordano, T. P. (2009). Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. Hepatology *49*, 116-123.
- Elliott, R. L., and Blobe, G. C. (2005). Role of transforming growth factor Beta in human cancer. J Clin Oncol *23*, 2078-2093.
- Emberley ED, Niu Y, Kliiewer EV, Murphy LC, and Watson PH (2003). Psoriasin (S100A7) expression is associated with poor outcome in oestrogen receptor negative invasive breast cancer. Clin Canc Res *9*, 2627-2631.

- Emmrich, J., Weber, I., Nausch, M., Sparmann, G., Koch, K., Seyfarth, M., Lohr, M., and Liebe, S. (1998). Immunohistochemical characterization of the pancreatic cellular infiltrate in normal pancreas, chronic pancreatitis and pancreatic carcinoma. Digestion *59*, 192-198.
- Ermolaeva, M. D. (2001). Synonymous codon usage in bacteria. Curr Issues Mol Biol *3*, 91-97.
- Esposito, I., Menicagli, M., Funel, N., Bergmann, F., Boggi, U., Mosca, F., Bevilacqua, G., and Campani, D. (2004). Inflammatory cells contribute to the generation of an angiogenic phenotype in pancreatic ductal adenocarcinoma. J Clin Pathol *57*, 630-636.
- Eue, I., Pietz, B., Storck, J., Klempt, M., and Sorg, C. (2000). Transendothelial migration of 27E10+ human monocytes. Int Immunol *12*, 1593-1604.
- Everhart, J., and Wright, D. (1995). Diabetes mellitus as a risk factor for pancreatic cancer. A meta-analysis. Jama *273*, 1605-1609.
- Fano G, Marsili V, Angelella P, Aisa MC, Giambanco I, and Donato R (1989). $S100a_0$ protein stimulates Ca^{2+} induced Ca^{2+} release from isolated sacroplasmic reticulum vesicles. FEBS Lett *255*, 381-384.
- Farrow, B., Albo, D., and Berger, D. H. (2008). The role of the tumor microenvironment in the progression of pancreatic cancer. J Surg Res *149*, 319-328.
- Farrow, B., and Evers, B. M. (2002). Inflammation and the development of pancreatic cancer. Surg Oncol *10*, 153-169.
- Feldmann, G., Habbe, N., Dhara, S., Bisht, S., Alvarez, H., Fendrich, V., Beaty, R., Mullendore, M., Karikari, C., Bardeesy, N., *et al.* (2008). Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer. Gut *57*, 1420-1430.
- Feng, G., Xu, X., Youssef, E. M., and Lotan, R. (2001). Diminished expression of S100A2, a putative tumor suppressor, at early stage of human lung carcinogenesis. Cancer Res *61*, 7999-8004.
- Fernando, R. I., Castillo, M. D., Litzinger, M., Hamilton, D. H., and Palena, C. (2011). IL-8 signaling plays a critical role in the epithelial-mesenchymal transition of human carcinoma cells. Cancer Res *71*, 5296-5306.
- Ferrara N, and Davis-Smyth T (1997). The biology of vascular endothelial growth factor. Endocr Rev *18*, 4-25.
- Ferrara N, and Keyt B (1997). Vascular endothelial growth factor: basic biology and clinical implications. Exs *79*, 209-232.
- Feurino, L. W., Fisher, W. E., Bharadwaj, U., Yao, Q., Chen, C., and Li, M. (2006). Current update of cytokines in pancreatic cancer: pathogenic mechanisms, clinical indication, and therapeutic values. Cancer Invest *24*, 696-703.
- Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M., *et al.* (2007). Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell *131*, 463-475.

- Fleming, J. B., Shen, G. L., Holloway, S. E., Davis, M., and Brekken, R. A. (2005). Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: justification for K-ras-directed therapy. Mol Cancer Res *3*, 413-423.
- Foell, D., Wittkowski, H., Ren, Z., Turton, J., Pang, G., Daebritz, J., Ehrchen, J., Heidemann, J., Borody, T., Roth, J., and Clancy, R. (2008). Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. J Pathol *216*, 183-192.
- Forssell, J., Oberg, A., Henriksson, M. L., Stenling, R., Jung, A., and Palmqvist, R. (2007). High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res *13*, 1472-1479.
- Friedl, P., and Alexander, S. (2011). Cancer invasion and the microenvironment: plasticity and reciprocity. Cell *147*, 992-1009.
- Friess, H., Guo, X. Z., Nan, B. C., Kleeff, J., and Buchler, M. W. (1999). Growth factors and cytokines in pancreatic carcinogenesis. Ann N Y Acad Sci *880*, 110-121.
- Friess, H., Yamanaka, Y., Buchler, M., Ebert, M., Beger, H. G., Gold, L. I., and Korc, M. (1993). Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. Gastroenterology *105*, 1846-1856.
- Frosch, M., Vogl, T., Waldherr, R., Sorg, C., Sunderkotter, C., and Roth, J. (2004). Expression of MRP8 and MRP14 by macrophages is a marker for severe forms of glomerulonephritis. J Leukoc Biol *75*, 198-206.
- Fujita, H., Ohuchida, K., Mizumoto, K., Egami, T., Miyoshi, K., Moriyama, T., Cui, L., Yu, J., Zhao, M., Manabe, T., and Tanaka, M. (2009). Tumor-stromal interactions with direct cell contacts enhance proliferation of human pancreatic carcinoma cells. Cancer Sci *100*, 2309-2317.
- Fukasawa, M., and Korc, M. (2004). Vascular endothelial growth factor-trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. Clin Cancer Res *10*, 3327-3332.
- Fukunaga, A., Miyamoto, M., Cho, Y., Murakami, S., Kawarada, Y., Oshikiri, T., Kato, K., Kurokawa, T., Suzuoki, M., Nakakubo, Y., *et al.* (2004). CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. Pancreas *28*, e26-31.
- Gaspar, N. J., Li, L., Kapoun, A. M., Medicherla, S., Reddy, M., Li, G., O'Young, G., Quon, D., Henson, M., Damm, D. L., *et al.* (2007). Inhibition of transforming growth factor beta signaling reduces pancreatic adenocarcinoma growth and invasiveness. Mol Pharmacol *72*, 152-161.
- Gebhardt, C., Nemeth, J., Angel, P., and Hess, J. (2006). S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol *72*, 1622-1631.
- Geoffroy-Perez B, Janin N, Ossian K, Lauge A, Croquette M, Griscelli C, Debre M, Bressac-de-Paillerets B, Aurias A, Stoppa-Lyonnet D, and Andrieu N (2001). Cancer risk in heterzygotes for ataxia-telangiectasia. Int J Cancer *93*, 288-293.
- Ghadirian, P., Lynch, H. T., and Krewski, D. (2003). Epidemiology of pancreatic cancer: an overview. Cancer Detect Prev *27*, 87-93.

- Ghaneh, P., Costello, E., and Neoptolemos, J. P. (2007). Biology and management of pancreatic cancer. Gut *56*, 1134-1152.
- Ghavami, S., Chitayat, S., Hashemi, M., Eshraghi, M., Chazin, W. J., Halayko, A. J., and Kerkhoff, C. (2009). S100A8/A9: a Janus-faced molecule in cancer therapy and tumorgenesis. Eur J Pharmacol *625*, 73-83.
- Ghavami, S., Eshragi, M., Ande, S. R., Chazin, W. J., Klonisch, T., Halayko, A. J., McNeill, K. D., Hashemi, M., Kerkhoff, C., and Los, M. S100A8/A9 induces autophagy and apoptosis via ROS-mediated cross-talk between mitochondria and lysosomes that involves BNIP3. Cell Res *20*, 314-331.
- Ghavami, S., Kerkhoff, C., Chazin, W. J., Kadkhoda, K., Xiao, W., Zuse, A., Hashemi, M., Eshraghi, M., Schulze-Osthoff, K., Klonisch, T., and Los, M. (2008a). S100A8/9 induces cell death via a novel, RAGE-independent pathway that involves selective release of Smac/DIABLO and Omi/HtrA2. Biochim Biophys Acta *1783*, 297-311.
- Ghavami, S., Rashedi, I., Dattilo, B. M., Eshraghi, M., Chazin, W. J., Hashemi, M., Wesselborg, S., Kerkhoff, C., and Los, M. (2008b). S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. J Leukoc Biol *83*, 1484-1492.
- Giardiello FM, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, and Offerhaus GJA (2000). Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology *119(6)*, 1447-1453.
- Giardiello FM, Offerhaus, G. J. A., Lee DH, Krush AJ, AC, T., SV, B., Kelley NC, and Hamilton SR (1993). Increased risk of thyroid and pancreatic carcinoma in familial adenomatous polyposis. Gut *34(10)*, 1394-1396.
- Gil-Bernabe, A. M., Ferjancic, S., Tlalka, M., Zhao, L., Allen, P. D., Im, J. H., Watson, K., Hill, S. A., Amirkhosravi, A., Francis, J. L., *et al.* (2012). Recruitment of monocytes/macrophages by tissue factor-mediated coagulation is essential for metastatic cell survival and premetastatic niche establishment in mice. Blood *119*, 3164-3175.
- Giovannucci, E. (2009). Vitamin D and cancer incidence in the Harvard cohorts. Ann Epidemiol *19*, 84-88.
- Glynne-Jones, E., Harper, M. E., Goddard, L., Eaton, C. L., Matthews, P. N., and Griffiths, K. (1994). Transforming growth factor beta 1 expression in benign and malignant prostatic tumors. Prostate *25*, 210-218.
- Goebeler, M., Roth, J., Burwinkel, F., Vollmer, E., Bocker, W., and Sorg, C. (1994). Expression and complex formation of S100-like proteins MRP8 and MRP14 by macrophages during renal allograft rejection. Transplantation *58*, 355-361.
- Gongoll S, Peters G, Mengel M, Piso P, Klempnauer J, Kriepe H, and Von Wasielewski R (2002). Prognostic significance of calcium binding protein S100A4 in colorectal cancer. Gastroenterology *123*, 1478-1484.
- Gordon, S., and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. Nat Rev Immunol *5*, 953-964.
- Goyette, J., and Geczy, C. L. (2010). Inflammation-associated S100 proteins: new mechanisms that regulate function. Amino Acids *41*, 821-842.

- Greer, J. B., Lynch, H. T., and Brand, R. E. (2009). Hereditary pancreatic cancer: a clinical perspective. Best Pract Res Clin Gastroenterol *23*, 159-170.
- Grigorian, M., Andresen, S., Tulchinsky, E., Kriajevska, M., Carlberg, C., Kruse, C., Cohn, M., Ambartsumian, N., Christensen, A., Selivanova, G., and Lukanidin, E. (2001). Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction. J Biol Chem *276*, 22699-22708.
- Grutzmann, R., McFaul, C., Bartsch, D. K., Sina-Frey, M., Rieder, H., Koch, R., McCarthy, E., Greenhalf, W., Neoptolemos, J. P., Saeger, H. D., and Pilarsky, C. (2004). No evidence for germline mutations of the LKB1/STK11 gene in familial pancreatic carcinoma. Cancer Lett *214*, 63-68.
- Gu, J., Yamamoto, H., Ogawa, M., Ngan, C. Y., Danno, K., Hemmi, H., Kyo, N., Takemasa, I., Ikeda, M., Sekimoto, M., and Monden, M. (2006). Hypoxia-induced up-regulation of angiopoietin-2 in colorectal cancer. Oncol Rep *15*, 779-783.
- Guerra, C., Collado, M., Navas, C., Schuhmacher, A. J., Hernandez-Porras, I., Canamero, M., Rodriguez-Justo, M., Serrano, M., and Barbacid, M. (2007). Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence. Cancer Cell *19*, 728-739.
- Gupta, S., Hussain, T., MacLennan, G. T., Fu, P., Patel, J., and Mukhtar, H. (2003). Differential expression of S100A2 and S100A4 during progression of human prostate adenocarcinoma. J Clin Oncol *21*, 106-112.
- Haddad, A., Kowdley, G. C., Pawlik, T. M., and Cunningham, S. C. (2011
-). Hereditary pancreatic and hepatobiliary cancers. Int J Surg Oncol *2011*, 154673.
- Haeno, H., Gonen, M., Davis, M. B., Herman, J. M., Iacobuzio-Donahue, C. A., and Michor, F. (2012). Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. Cell *148*, 362-375.
- Hahn SA, and et al (1996). DCP4, a candidate tumour suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.
- Hahn, S. A., Greenhalf, B., Ellis, I., Sina-Frey, M., Rieder, H., Korte, B., Gerdes, B., Kress, R., Ziegler, A., Raeburn, J. A., *et al.* (2003). BRCA2 germline mutations in familial pancreatic carcinoma. J Natl Cancer Inst *95*, 214-221.
- Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.
- Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
- Harpio, R., and Einarsson, R. (2004). S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. Clin Biochem *37*, 512-518.
- Harvey SH, Hurd TC, Markus G, Martinick MI, Penetrante RM, Tan D, Venkatamaran P, DeSouza N, Sait SNJ, Driscoll DL, and Gibbs JF (2003). Evaluation of urinary palsminogen activator, its receptor, matrix metalloproteinase-9, and von-Willebrand factor in pancreatic cancer. Clin Canc Res *9*, 4935-4943.

- Hayden, M. S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev *18*, 2195-2224.
- He, K., Xu, T., and Goldkorn, A. (2011). Cancer cells cyclically lose and regain drug-resistant highly tumorigenic features characteristic of a cancer stem-like phenotype. Mol Cancer Ther *10*, 938-948.
- Heino J (1996). Biology of tumour cell invasion: interplay of cell adhesion and matrix degradation. Int J Cancer *65*, 717-722.
- Heizmann CW, and Cox JA (1998). New perspectives on S100 proteins: a multi functional Ca(2+), Zn (2+) and Cu (2+) binding protein family. Biometals *11*, 383-397.
- Helfman, D. M., Kim, E. J., Lukanidin, E., and Grigorian, M. (2005). The metastasis associated protein S100A4: role in tumour progression and metastasis. Br J Cancer *92*, 1955-1958.
- Hermani, A., De Servi, B., Medunjanin, S., Tessier, P. A., and Mayer, D. (2006). S100A8 and S100A9 activate MAP kinase and NF-[kappa]B signaling pathways and trigger translocation of RAGE in human prostate cancer cells. Experimental Cell Research *312*, 184-197.
- Hermani, A., Hess, J., De Servi, B., Medunjanin, S., Grobholz, R., Trojan, L., Angel, P., and Mayer, D. (2005). Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. Clin Cancer Res *11*, 5146-5152.
- Hezel, A. F., Kimmelman, A. C., Stanger, B. Z., Bardeesy, N., and DePinho, R. A. (2006). Genetics and biology of pancreatic ductal adenocarcinoma
- 10.1101/gad.1415606. Genes Dev *20*, 1218-1249.
- Hidalgo, M., and Maitra, A. (2009). The hedgehog pathway and pancreatic cancer. N Engl J Med *361*, 2094-2096.
- Hill, R., Song, Y., Cardiff, R. D., and Van Dyke, T. (2005). Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell *123*, 1001-1011.
- Hingorani, S. R., Petricoin, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A., *et al.* (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell *4*, 437-450.
- Hingorani, S. R., and Tuveson, D. A. (2003). Ras redux: rethinking how and where Ras acts. Curr Opin Genet Dev *13*, 6-13.
- Hirano, T., Shino, Y., Saito, T., Komoda, F., Okutomi, Y., Takeda, A., Ishihara, T., Yamaguchi, T., Saisho, H., and Shirasawa, H. (2002). Dominant negative MEKK1 inhibits survival of pancreatic cancer cells. Oncogene *21*, 5923-5928.
- Hiratsuka, S., Watanabe, A., Aburatani, H., and Maru, Y. (2006). Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. Nat Cell Biol *8*, 1369-1375.
- Hochegger, H., Takeda, S., and Hunt, T. (2008). Cyclin-dependent kinases and cell-cycle transitions: does one fit all? Nat Rev Mol Cell Biol *9*, 910-916.
- Howe GR, and Burch JD (1996). Nutrition and pancreatic cancer. Cancer Causes Control *7*, 69-82.

- Howes N, Greehalf W, Stocken DD, and Neoptolemos JP (2004). Cationic trypsinogen mutations and pancreatitis. Gastroenterol Clin N Am *33*, 767-787.
- Howes, N., Lerch, M. M., Greenhalf, W., Stocken, D. D., Ellis, I., Simon, P., Truninger, K., Ammann, R., Cavallini, G., Charnley, R. M., *et al.* (2004). Clinical and genetic characteristics of hereditary pancreatitis in Europe. Clin Gastroenterol Hepatol *2*, 252-261.
- Howes, N., and Neoptolemos, J. P. (2002). Risk of pancreatic ductal adenocarcinoma in chronic pancreatitis. Gut *51*, 765-766.
- Hruban, R. H., and Adsay, N. V. (2009). Molecular classification of neoplasms of the pancreas. Hum Pathol *40*, 612-623.
- Hruban, R. H., Adsay, N. V., Albores-Saavedra, J., Compton, C., Garrett, E. S., Goodman, S. N., Kern, S. E., Klimstra, D. S., Kloppel, G., Longnecker, D. S., *et al.* (2001). Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol *25*, 579-586.
- Hruban, R. H., Goggins, M., Parsons, J., and Kern, S. E. (2000). Progression model for pancreatic cancer. Clin Cancer Res *6*, 2969-2972.
- Hruban, R. H., Maitra, A., Schulick, R., Laheru, D., Herman, J., Kern, S. E., and Goggins, M. (2008). Emerging molecular biology of pancreatic cancer. Gastrointest Cancer Res *2*, S10-15.
- Hruban, R. H., van Mansfeld, A. D., Offerhaus, G. J., van Weering, D. H., Allison, D. C., Goodman, S. N., Kensler, T. W., Bose, K. K., Cameron, J. L., and Bos, J. L. (1993). K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. Am J Pathol *143*, 545-554.
- Hruban, R. K. D., Pitman MB (2006). Tumors of the Pancreas (Washington: Armed Forces Inst. Pathology).
- Huang, C., Yang, G., Jiang, T., Zhu, G., Li, H., and Qiu, Z. (2011). The effects and mechanisms of blockage of STAT3 signaling pathway on IL-6 inducing EMT in human pancreatic cancer cells in vitro. Neoplasma *58*, 396-405.
- Huang, S. S., and Huang, J. S. (2005). TGF-beta control of cell proliferation. J Cell Biochem *96*, 447-462.
- Huxley, R., Ansary-Moghaddam, A., Berrington de Gonzalez, A., Barzi, F., and Woodward, M. (2005). Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. Br J Cancer *92*, 2076-2083.
- Hwang, R. F., Moore, T., Arumugam, T., Ramachandran, V., Amos, K. D., Rivera, A., Ji, B., Evans, D. B., and Logsdon, C. D. (2008). Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res *68*, 918-926.
- Iacobuzio-Donahue, C. A. (2011). Genetic evolution of pancreatic cancer: lessons learnt from the pancreatic cancer genome sequencing project. Gut.
- Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J., and Kern, S. E. (2002a). The desmoplastic response to infiltrating breast carcinoma: gene expression at the site of primary invasion and implications for comparisons between tumor types. Cancer Res *62*, 5351-5357.

- Iacobuzio-Donahue, C. A., Fu, B., Yachida, S., Luo, M., Abe, H., Henderson, C. M., Vilardell, F., Wang, Z., Keller, J. W., Banerjee, P., *et al.* (2009). DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. J Clin Oncol *27*, 1806-1813.
- Iacobuzio-Donahue, C. A., Ryu, B., Hruban, R. H., and Kern, S. E. (2002b). Exploring the host desmoplastic response to pancreatic carcinoma: gene expression of stromal and neoplastic cells at the site of primary invasion. Am J Pathol *160*, 91-99.
- IARC (2008). http://www.iarc.fr/.
- Ichikawa, M., Williams, R., Wang, L., Vogl, T., and Srikrishna, G. (2011). S100A8/A9 activate key genes and pathways in colon tumor progression. Mol Cancer Res *9*, 133-148.
- Ikura M, Osawa M, and Ames JB (2002). The role of calcium-binding proteins in the control of transcription: structure to function. BioEssays *24*, 625-636.
- Imhof, B. A., and Aurrand-Lions, M. (2006). Angiogenesis and inflammation face off. Nat Med *12*, 171-172.
- Iodice, S., Gandini, S., Maisonneuve, P., and Lowenfels, A. B. (2008). Tobacco and the risk of pancreatic cancer: a review and meta-analysis. Langenbecks Arch Surg *393*, 535-545.
- Ishiwata, T., Friess, H., Buchler, M. W., Lopez, M. E., and Korc, M. (1998). Characterization of keratinocyte growth factor and receptor expression in human pancreatic cancer. Am J Pathol *153*, 213-222.
- Itakura, J., Ishiwata, T., Shen, B., Kornmann, M., and Korc, M. (2000). Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer. Int J Cancer *85*, 27-34.
- Iwamura, T., Katsuki, T., and Ide, K. (1987). Establishment and characterization of a human pancreatic cancer cell line (SUIT-2) producing carcinoembryonic antigen and carbohydrate antigen 19-9. Jpn J Cancer Res *78*, 54-62.
- Jaffee, E. M., Hruban, R. H., Canto, M., and Kern, S. E. (2002). Focus on pancreas cancer. Cancer Cell *2*, 25-28.
- Jang, J. Y., Park, Y. C., Song, Y. S., Lee, S. E., Hwang, D. W., Lim, C. S., Lee, H. E., Kim, W. H., and Kim, S. W. (2009). Increased K-ras mutation and expression of S100A4 and MUC2 protein in the malignant intraductal papillary mucinous tumor of the pancreas. J Hepatobiliary Pancreat Surg *16*, 668-674.
- Javle, M. M., Shroff, R. T., Xiong, H., Varadhachary, G. A., Fogelman, D., Reddy, S. A., Davis, D., Zhang, Y., Wolff, R. A., and Abbruzzese, J. L. (2010). Inhibition of the mammalian target of rapamycin (mTOR) in advanced pancreatic cancer: results of two phase II studies. BMC Cancer *10*, 368.
- Jerpseth, M., Jerpseth, B., Briester, L., and Greener (1998). Strategies *11*, 3-4.
- Jiao, L., Mitrou, P. N., Reedy, J., Graubard, B. I., Hollenbeck, A. R., Schatzkin, A., and Stolzenberg-Solomon, R. (2009). A combined healthy lifestyle score and risk of pancreatic cancer in a large cohort study. Arch Intern Med *169*, 764-770.
- Jimeno, A., Feldmann, G., Suarez-Gauthier, A., Rasheed, Z., Solomon, A., Zou, G. M., Rubio-Viqueira, B., Garcia-Garcia, E., Lopez-Rios, F., Matsui, W., *et al.*

(2009). A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol Cancer Ther *8*, 310-314.

- Johnson DG (2000). The paradox of E2F1: Oncogene and tumour suppressor gene. Mol Carcinogenesis *27*, 151-157.
- Jones, S., Zhang, X., Parsons, D. W., Lin, J. C., Leary, R. J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A., *et al.* (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science *321*, 1801-1806.
- Jorgensen, M. T., Mortensen, M. B., Gerdes, A. M., and De Muckadell, O. B. (2008). Familial pancreatic cancer. Scand J Gastroenterol *43*, 387-397.
- Joyce, J. A., and Pollard, J. W. (2009). Microenvironmental regulation of metastasis. Nat Rev Cancer *9*, 239-252.
- Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. Nat Rev Cancer *6*, 392-401.
- Kane, D., Roth, J., Frosch, M., Vogl, T., Bresnihan, B., and FitzGerald, O. (2003). Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. Arthritis Rheum *48*, 1676-1685.
- Kane, R. C., Farrell, A. T., Saber, H., Tang, S., Williams, G., Jee, J. M., Liang, C., Booth, B., Chidambaram, N., Morse, D., *et al.* (2006). Sorafenib for the treatment of advanced renal cell carcinoma. Clin Cancer Res *12*, 7271-7278.
- Kang HM, Choi KS, Kassam G, Fitzpatrick SL, Louie P, and Waisman DM (1998). The p11 subunit of the annexin II tetramer plays a key role in the stimulation of tPA dependant plasminogen activation. Biochemistry *37*, 16958-16966.
- Kayed, H., Kleeff, J., Keleg, S., Guo, J., Ketterer, K., Berberat, P. O., Giese, N., Esposito, I., Giese, T., Buchler, M. W., and Friess, H. (2004). Indian hedgehog signaling pathway: expression and regulation in pancreatic cancer. Int J Cancer *110*, 668-676.
- Kennedy, R. D., Gorski, J. J., Quinn, J. E., Stewart, G. E., James, C. R., Moore, S., Mulligan, K., Emberley, E. D., Lioe, T. F., Morrison, P. J., *et al.* (2005). BRCA1 and c-Myc associate to transcriptionally repress psoriasin, a DNA damage-inducible gene. Cancer Res *65*, 10265-10272.
- Kerkhoff, C., Klempt, M., and Sorg, C. (1998). Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). Biochim Biophys Acta *1448*, 200-211.
- Khasawneh, J., Schulz, M. D., Walch, A., Rozman, J., Hrabe de Angelis, M., Klingenspor, M., Buck, A., Schwaiger, M., Saur, D., Schmid, R. M., *et al.* (2009). Inflammation and mitochondrial fatty acid beta-oxidation link obesity to early tumor promotion. Proc Natl Acad Sci U S A *106*, 3354-3359.
- Kim, B. G., Li, C., Qiao, W., Mamura, M., Kasprzak, B., Anver, M., Wolfraim, L., Hong, S., Mushinski, E., Potter, M., *et al.* (2006). Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature *441*, 1015-1019.
- Kim E. Barrett, Susan M. Barman, Scott Boitano, and Brooks, H. L. (2010). Ganong's Review of Medical Physiology. *23rd Edition.*
- Kim SJ, Shiba E, Kobari T, Yayoi E, Furukawa J, Takatsuka Y, Shin E, Koyama H, Inaji H, and Taki S (1998). Prognostic impact of urokinase-type plasminogen

activator (PA), PA inhibitor type 1, and tissue type PA antigen levels in node negative breast cancer: a prospective study on multicentre basis. Clin Canc Res *4*, 177-182.

- Kim, Y. J., Kim, M. A., Im, S. A., Kim, T. M., Kim, D. W., Yang, H. K., Heo, D. S., Lee, K. U., Choe, K. J., Kim, N. K., *et al.* (2008). Metastasis-associated protein S100A4 and p53 predict relapse in curatively resected stage III and IV (M0) gastric cancer. Cancer Invest *26*, 152-158.
- Kimura, M., Yoshida, Y., Narita, M., Takenaga, K., Takenouchi, T., Yamaguchi, T., Saisho, H., Sakiyama, S., and Tagawa, M. (1999). Acquired immunity in nude mice induced by expression of the IL-2 or IL-4 gene in human pancreatic carcinoma cells and anti-tumor effect generated by in vivo gene transfer using retrovirus. Int J Cancer *82*, 549-555.
- Kinoshita, S., Wagatsuma, Y., and Okada, M. (2007). Geographical distribution for malignant neoplasm of the pancreas in relation to selected climatic factors in Japan. Int J Health Geogr *6*, 34.
- Kitamura, T., Kometani, K., Hashida, H., Matsunaga, A., Miyoshi, H., Hosogi, H., Aoki, M., Oshima, M., Hattori, M., Takabayashi, A., *et al.* (2007). SMAD4-deficient intestinal tumors recruit CCR1+ myeloid cells that promote invasion. Nat Genet *39*, 467-475.
- Kleef J, Ishiwata T, Kumbasar A, Friess H, Buchler M, Lander AD, and Korc M (1998). The cell surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer. J Clin Invest *102*, 1662-1673.
- Kleeff, J., Beckhove, P., Esposito, I., Herzig, S., Huber, P. E., Lohr, J. M., and Friess, H. (2007). Pancreatic cancer microenvironment. Int J Cancer.
- Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJA, Griffin C, Cameron JL, Yeo CJ, Kern S, and Hruban RH (2004). Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. Canc Res *64*, 2634-2638.
- Knudson A (1978). Mutation and cancer: a statistical study of retinoblastoma. Proc Natl Acad Sci USA *68*, 820-823.
- Kobrin MS, Yamanaka Y, Friess H, Lopez ME, and Korc M (1993). Aberrant expression of type 1 fibroblast growth factor receptor in human pancreatic adenocarcinomas. Canc Res *53*, 4741-4744.
- Komatsu, K., Kobune-Fujiwara, Y., Andoh, A., Ishiguro, S., Hunai, H., Suzuki, N., Kameyama, M., Murata, K., Miyoshi, J., Akedo, H., *et al.* (2000). Increased expression of S100A6 at the invading fronts of the primary lesion and liver metastasis in patients with colorectal adenocarcinoma. Br J Cancer *83*, 769-774.
- Kong, J. P., Ding, F., Zhou, C. N., Wang, X. Q., Miao, X. P., Wu, M., and Liu, Z. H. (2004). Loss of myeloid-related proteins 8 and myeloid-related proteins 14 expression in human esophageal squamous cell carcinoma correlates with poor differentiation. World J Gastroenterol *10*, 1093-1097.
- Koninger, J., Giese, T., di Mola, F. F., Wente, M. N., Esposito, I., Bachem, M. G., Giese, N. A., Buchler, M. W., and Friess, H. (2004). Pancreatic tumor cells influence the composition of the extracellular matrix. Biochem Biophys Res Commun *322*, 943-949.

- Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, and Kallioiemi OP (1998). Tissue microarrays for high throughput molecular profiling of tumour specimens. Nature Med *4*, 844-847.
- Korc, M. (2003). Pathways for aberrant angiogenesis in pancreatic cancer. Mol Cancer *2*, 8.
- Kordes, C., Brookmann, S., Haussinger, D., and Klonowski-Stumpe, H. (2005). Differential and synergistic effects of platelet-derived growth factor-BB and transforming growth factor-beta1 on activated pancreatic stellate cells. Pancreas *31*, 156-167.
- Kornmann M, Beger HG, and Korc M (1998). Role of fibroblast growth factor and their receptors in pancreatic cancer and chronic pancreatitis. Pancreas *17*, 169-175.
- Kornmann M, Ishiwata T, Beger HG, and Korc M (1997). Fibroblast growth factor-5 stimulates mitogenic signalling and is overexpressed in human pancreatic cancer: evidence for autocrine and paracrine actions. Oncogene *15*, 1417-1424.
- Koukourakis, M. I., Giatromanolaki, A., Brekken, R. A., Sivridis, E., Gatter, K. C., Harris, A. L., and Sage, E. H. (2003). Enhanced expression of SPARC/osteonectin in the tumor-associated stroma of non-small cell lung cancer is correlated with markers of hypoxia/acidity and with poor prognosis of patients. Cancer Res *63*, 5376-5380.
- Krantz, S. B., Shields, M. A., Dangi-Garimella, S., Cheon, E. C., Barron, M. R., Hwang, R. F., Rao, M. S., Grippo, P. J., Bentrem, D. J., and Munshi, H. G. (2011). MT1-MMP cooperates with Kras(G12D) to promote pancreatic fibrosis through increased TGF-beta signaling. Mol Cancer Res *9*, 1294-1304.
- Kresse, H., and Schonherr, E. (2001). Proteoglycans of the extracellular matrix and growth control. J Cell Physiol *189*, 266-274.
- Kretsinger RH, and Nockolds CE (1973). carp muscle calcium binding protein. II. Structure determination and general description. J Biol Chem *248*, 3313-3326.
- Kubuschok, B., Xie, X., Jesnowski, R., Preuss, K. D., Romeike, B. F., Neumann, F., Regitz, E., Pistorius, G., Schilling, M., Scheunemann, P., *et al.* (2004). Expression of cancer testis antigens in pancreatic carcinoma cell lines, pancreatic adenocarcinoma and chronic pancreatitis. Int J Cancer *109*, 568-575.
- Kuperwasser, C., Chavarria, T., Wu, M., Magrane, G., Gray, J. W., Carey, L., Richardson, A., and Weinberg, R. A. (2004). Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A *101*, 4966-4971.
- Kurahara, H., Shinchi, H., Mataki, Y., Maemura, K., Noma, H., Kubo, F., Sakoda, M., Ueno, S., Natsugoe, S., and Takao, S. (2009). Significance of M2-Polarized Tumor-Associated Macrophage in Pancreatic Cancer. J Surg Res.
- Kurahara, H., Takao, S., Kuwahata, T., Nagai, T., Ding, Q., Maeda, K., Shinchi, H., Mataki, Y., Maemura, K., Matsuyama, T., and Natsugoe, S. (2012a). Clinical Significance of Folate Receptor beta-expressing Tumor-associated Macrophages in Pancreatic Cancer. Ann Surg Oncol.
- Kurahara, H., Takao, S., Maemura, K., Mataki, Y., Kuwahata, T., Maeda, K., Ding, Q., Sakoda, M., Iino, S., Ishigami, S., *et al.* (2012b). Epithelial-

mesenchymal transition and mesenchymal-epithelial transition via regulation of ZEB-1 and ZEB-2 expression in pancreatic cancer. J Surg Oncol.

- Kuwada, Y., Sasaki, T., Morinaka, K., Kitadai, Y., Mukaida, N., and Chayama, K. (2003). Potential involvement of IL-8 and its receptors in the invasiveness of pancreatic cancer cells. Int J Oncol *22*, 765-771.
- Kuwahara K, Sasaki T, Kuwada Y, Murakami M, Yamasaki S, and Chayama K (2003). Expressions of angiogenic factors in pancreatic ductal carcinoma: correlative study with clinicopathologic parameters and patient survival. Pancreas *26*, 344-349.
- Lamagna, C., Aurrand-Lions, M., and Imhof, B. A. (2006). Dual role of macrophages in tumor growth and angiogenesis. J Leukoc Biol *80*, 705-713.
- Latchford, A., Greenhalf, W., Vitone, L. J., Neoptolemos, J. P., Lancaster, G. A., and Phillips, R. K. (2006). Peutz-Jeghers syndrome and screening for pancreatic cancer. Br J Surg *93*, 1446-1455.
- Lee, P. N., and Hamling, J. (2009a). The relation between smokeless tobacco and cancer in Northern Europe and North America. A commentary on differences between the conclusions reached by two recent reviews. BMC Cancer *9*, 256.
- Lee, P. N., and Hamling, J. (2009b). Systematic review of the relation between smokeless tobacco and cancer in Europe and North America. BMC Med *7*, 36.
- Lelbovitz, A., Wright, W. C., Pathak, S., Siciliano, M. J., and Daniels, W. P. (1979). Detection and analysis of a glucose 6-phosphate dehydrogenase phenotype B cell line contamination. J Natl Cancer Inst *63*, 635-645.
- Lemmens, V. E., Bosscha, K., van der Schelling, G., Brenninkmeijer, S., Coebergh, J. W., and de Hingh, I. H. (2011
-). Improving outcome for patients with pancreatic cancer through centralization. Br J Surg *98*, 1455-1462.
- Leukert, N., Vogl, T., Strupat, K., Reichelt, R., Sorg, C., and Roth, J. (2006). Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity. J Mol Biol *359*, 961-972.
- Leung HY, Gullick WJ, and Lemoine NR (1994). Expression and functional activity of fibroblast growth factors and their receptors in human pancreatic cancer. Int J Cancer *59*, 667-675.
- Lewis, C., and Murdoch, C. (2005). Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol *167*, 627-635.
- Lewis, C. E., and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. Cancer Res *66*, 605-612.
- Lewthwaite, J., George, R., Lund, P. A., Poole, S., Tormay, P., Sharp, L., Coates, A. R., and Henderson, B. (2002). Rhizobium leguminosarum chaperonin 60.3, but not chaperonin 60.1, induces cytokine production by human monocytes: activity is dependent on interaction with cell surface CD14. Cell Stress Chaperones *7*, 130-136.
- Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. (2000). Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med *343*, 78-85.

- Lieber, M., Mazzetta, J., Nelson-Rees, W., Kaplan, M., and Todaro, G. (1975). Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. Int J Cancer *15*, 741-747.
- Lim, K. H., O'Hayer, K., Adam, S. J., Kendall, S. D., Campbell, P. M., Der, C. J., and Counter, C. M. (2006). Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells. Curr Biol *16*, 2385-2394.
- Lin, E. Y., Gouon-Evans, V., Nguyen, A. V., and Pollard, J. W. (2002). The macrophage growth factor CSF-1 in mammary gland development and tumor progression. J Mammary Gland Biol Neoplasia *7*, 147-162.
- Lin, E. Y., Li, J. F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., Qian, H., Xue, X. N., and Pollard, J. W. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res *66*, 11238-11246.
- Lin, E. Y., Nguyen, A. V., Russell, R. G., and Pollard, J. W. (2001). Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med *193*, 727-740.
- Lin, E. Y., and Pollard, J. W. (2004). Macrophages: modulators of breast cancer progression. Novartis Found Symp *256*, 158-168; discussion 168-172, 259-169.
- Lin, G., Zeng, Z., Wang, X., Wu, Z., Wang, J., Wang, C., Sun, Q., Chen, Y., and Quan, H. (2012). Cholecystectomy and risk of pancreatic cancer: a meta-analysis of observational studies. Cancer Causes Control *23*, 59-67.
- Lin Y, Tamokoshi A, Kawamura T, Inaba Y, Kikuchi S, Motohashi Y, Kurosawa M, and Ohno Y (2002). Risk of pancreatic cancer in relation to alcohol drinking, coffee consumption and medical history: findings from the Japan collaborative cohort study for evaluation of cancer risk. Int J Cancer *99*, 742-746.
- Lindkvist, B., Johansen, D., Borgstrom, A., and Manjer, J. (2008). A prospective study of Helicobacter pylori in relation to the risk for pancreatic cancer. BMC Cancer *8*, 321.
- Lines, K. E., Chelala, C., Dmitrovic, B., Wijesuriya, N., Kocher, H. M., Marshall, J. F., and Crnogorac-Jurcevic, T. (2012). S100P-Binding Protein, S100PBP, Mediates Adhesion through Regulation of Cathepsin Z in Pancreatic Cancer Cells. Am J Pathol *180*, 1485-1494.
- Liotta, L. A., and Kohn, E. C. (2001). The microenvironment of the tumour-host interface. Nature *411*, 375-379.
- Liptay, S., Weber, C. K., Ludwig, L., Wagner, M., Adler, G., and Schmid, R. M. (2003). Mitogenic and antiapoptotic role of constitutive NF-kappaB/Rel activity in pancreatic cancer. Int J Cancer *105*, 735-746.
- Liu, D., Rudland, P. S., Sibson, D. R., Platt-Higgins, A., and Barraclough, R. (2000). Expression of calcium-binding protein S100A2 in breast lesions. Br J Cancer *83*, 1473-1479.
- Liu, Y., Elsawa, S. F., and Almada, L. L. (2010). Primers on molecular pathways - cycling toward pancreatic cancer. Pancreatology *10*, 6-13.
- Locati, M., Deuschle, U., Massardi, M. L., Martinez, F. O., Sironi, M., Sozzani, S., Bartfai, T., and Mantovani, A. (2002). Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. J Immunol *168*, 3557-3562.

- Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*, 487-501.
- Lonardo, E., Frias-Aldeguer, J., Hermann, P., and Heeschen, C. (2012). Pancreatic stellate cells form a niche for cancer stem cells and promote their self-renewal and invasiveness. Cell Cycle *11*.
- Loser, K., Vogl, T., Voskort, M., Lueken, A., Kupas, V., Nacken, W., Klenner, L., Kuhn, A., Foell, D., Sorokin, L., *et al.* (2010). The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8+ T cells. Nat Med *16*, 713-717.
- Lowenfels, A. B., and Maisonneuve, P. (2006). Epidemiology and risk factors for pancreatic cancer. Best Pract Res Clin Gastroenterol *20*, 197-209.
- Lowery, M. A., and O'Reilly, E. M. (2011). Pancreatic cancer: the role of molecular markers in diagnosis and management. Clin Adv Hematol Oncol *9*, 900-908.
- Lu, Z., Friess, H., Graber, H. U., Guo, X., Schilling, M., Zimmermann, A., Korc, M., and Buchler, M. W. (1997). Presence of two signaling TGF-beta receptors in human pancreatic cancer correlates with advanced tumor stage. Dig Dis Sci *42*, 2054-2063.
- Luo, J., Iwasaki, M., Inoue, M., Sasazuki, S., Otani, T., Ye, W., and Tsugane, S. (2007). Body mass index, physical activity and the risk of pancreatic cancer in relation to smoking status and history of diabetes: a large-scale population-based cohort study in Japan--the JPHC study. Cancer Causes Control *18*, 603-612.
- Luster, A. D. (1998). Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med *338*, 436-445.
- Luttges, J., Galehdari, H., Brocker, V., Schwarte-Waldhoff, I., Henne-Bruns, D., Kloppel, G., Schmiegel, W., and Hahn, S. A. (2001). Allelic Loss Is Often the First Hit in the Biallelic Inactivation of the p53 and DPC4 Genes During Pancreatic Carcinogenesis. Am J Pathol *158*, 1677-1683.
- Lynch, C. C., Hikosaka, A., Acuff, H. B., Martin, M. D., Kawai, N., Singh, R. K., Vargo-Gogola, T. C., Begtrup, J. L., Peterson, T. E., Fingleton, B., *et al.* (2005). MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. Cancer Cell *7*, 485-496.
- Lynch HT, Fitzsimmons ML, Smyrk TC, Lanspa SJ, Watson P, McClellan J, Lynch JF, Department of Preventive Medicine Public Health, Creighton University School of Medicine, Omaha, and Nebraska (1990). Familial pancreatic cancer: clinicopathologic study of 18 nuclear families. Am J Gastroenterol *85*, 54-60.
- Lynch, H. T., Lanspa, S. J., Fitzgibbons, R. J., Jr., Smyrk, T., Fitzsimmons, M. L., and McClellan, J. (1989). Familial pancreatic cancer (Part 1): Genetic pathology review. Nebr Med J *74*, 109-112.
- lyWyckoff, J. B., Wang, Y., Lin, E. Y., Li, J. F., Goswami, S., Stanley, E. R., Segall, J. E., Pollard, J. W., and Condeelis, J. (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res *67*, 2649-2656.
- Maelandsmo, G. M., Florenes, V. A., Mellingsaeter, T., Hovig, E., Kerbel, R. S., and Fodstad, O. (1997). Differential expression patterns of S100A2, S100A4 and

S100A6 during progression of human malignant melanoma. Int J Cancer *74*, 464-469.

- Mahadevan, D., and Von Hoff, D. D. (2007). Tumor-stroma interactions in pancreatic ductal adenocarcinoma. Mol Cancer Ther *6*, 1186-1197.
- Maitra, A., Adsay, N. V., Argani, P., Iacobuzio-Donahue, C., De Marzo, A., Cameron, J. L., Yeo, C. J., and Hruban, R. H. (2003). Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. Mod Pathol *16*, 902-912.
- Maitra, A., Fukushima, N., Takaori, K., and Hruban, R. H. (2005). Precursors to invasive pancreatic cancer. Adv Anat Pathol *12*, 81-91.
- Maitra, A., and Hruban, R. H. (2008). Pancreatic cancer. Annu Rev Pathol *3*, 157-188.
- Maitra, A., Kern, S. E., and Hruban, R. H. (2006). Molecular pathogenesis of pancreatic cancer. Best Practice & Research Clinical Gastroenterology *20*, 211-226.
- Malats, N., Porta, M., Corominas, J. M., Pinol, J. L., Rifa, J., and Real, F. X. (1997). Ki-ras mutations in exocrine pancreatic cancer: association with clinico-pathological characteristics and with tobacco and alcohol consumption. PANK-ras I Project Investigators. Int J Cancer *70*, 661-667.
- Malka, D., Hammel, P., Maire, F., Rufat, P., Madeira, I., Pessione, F., Levy, P., and Ruszniewski, P. (2002). Risk of pancreatic adenocarcinoma in chronic pancreatitis. Gut *51*, 849-852.
- Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends Biochem Sci *30*, 630-641.
- Malvezzi, M., Bertuccio, P., Levi, F., La Vecchia, C., and Negri, E. (2012). European cancer mortality predictions for the year 2012. Ann Oncol *23*, 1044-1052.
- Manitz, M. P., Horst, B., Seeliger, S., Strey, A., Skryabin, B. V., Gunzer, M., Frings, W., Schonlau, F., Roth, J., Sorg, C., and Nacken, W. (2003). Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. Mol Cell Biol *23*, 1034-1043.
- Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol *25*, 677-686.
- Marchesi, F., Grizzi, F., Laghi, L., Mantovani, A., and Allavena, P. (2012). Molecular Mechanisms of Pancreatic Cancer Dissemination: The Role of the Chemokine System. Curr Pharm Des.
- Marenholz, I., Heizmann, C. W., and Fritz, G. (2004). S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun *322*, 1111-1122.
- Markowitz SD, and Roberts AB (1996). Tumour suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev *7*, 93-102.
- Masamune, A., Kikuta, K., Watanabe, T., Satoh, K., Hirota, M., Hamada, S., and Shimosegawa, T. (2009a). Fibrinogen induces cytokine and collagen production in pancreatic stellate cells. Gut *58*, 550-559.

- Masamune, A., Kikuta, K., Watanabe, T., Satoh, K., Hirota, M., and Shimosegawa, T. (2008a). Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer. Am J Physiol Gastrointest Liver Physiol *295*, G709-717.
- Masamune, A., Kikuta, K., Watanabe, T., Satoh, K., Satoh, A., and Shimosegawa, T. (2008b). Pancreatic stellate cells express Toll-like receptors. J Gastroenterol *43*, 352-362.
- Masamune, A., and Shimosegawa, T. (2009). Signal transduction in pancreatic stellate cells. J Gastroenterol *44*, 249-260.
- Masamune, A., Watanabe, T., Kikuta, K., and Shimosegawa, T. (2009b). Roles of pancreatic stellate cells in pancreatic inflammation and fibrosis. Clin Gastroenterol Hepatol *7*, S48-54.
- Massague, J. (1998). TGF-beta signal transduction. Annu Rev Biochem *67*, 753-791.
- Massague J, Blain SW, and Lo RS (2000). TGF-β signalling in growth control, cancer and heritable disrders. Cell *103*, 295-309.
- Massague, J., Blain, S. W., and Lo, R. S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. Cell *103*, 295-309.
- Mathisen, B., Lindstad, R. I., Hansen, J., El-Gewely, S. A., Maelandsmo, G. M., Hovig, E., Fodstad, O., Loennechen, T., and Winberg, J. O. (2003). S100A4 regulates membrane induced activation of matrix metalloproteinase-2 in osteosarcoma cells. Clin Exp Metastasis *20*, 701-711.
- Matsuo, Y., Sawai, H., Funahashi, H., Takahashi, H., Sakamoto, M., Yamamoto, M., Okada, Y., Hayakawa, T., and Manabe, T. (2004). Enhanced angiogenesis due to inflammatory cytokines from pancreatic cancer cell lines and relation to metastatic potential. Pancreas *28*, 344-352.
- Matsuo, Y., Takeyama, H., and Guha, S. (2012). Cytokine Network: New Targeted Therapy for Pancreatic Cancer. Curr Pharm Des.
- Matthaei, H., Schulick, R. D., Hruban, R. H., and Maitra, A. (2011). Cystic precursors to invasive pancreatic cancer. Nat Rev Gastroenterol Hepatol *8*, 141-150.
- Matthaios, D., Zarogoulidis, P., Balgouranidou, I., Chatzaki, E., and Kakolyris, S. (2011). Molecular pathogenesis of pancreatic cancer and clinical perspectives. Oncology *81*, 259-272.
- Meeker, A. K., and De Marzo, A. M. (2004). Recent advances in telomere biology: implications for human cancer. Curr Opin Oncol *16*, 32-38.
- Mihaljevic, A. L., Michalski, C. W., Friess, H., and Kleeff, J. (2010
-). Molecular mechanism of pancreatic cancer--understanding proliferation, invasion, and metastasis. Langenbecks Arch Surg *395*, 295-308.
- Mischke, D., Korge, B. P., Marenholz, I., Volz, A., and Ziegler, A. (1996). Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. J Invest Dermatol *106*, 989-992.
- Mohamed, M. M., and Sloane, B. F. (2006). Cysteine cathepsins: multifunctional enzymes in cancer. Nat Rev Cancer *6*, 764-775.

- Moon, A., Yong, H. Y., Song, J. I., Cukovic, D., Salagrama, S., Kaplan, D., Putt, D., Kim, H., Dombkowski, A., and Kim, H. R. (2008). Global gene expression profiling unveils S100A8/A9 as candidate markers in H-ras-mediated human breast epithelial cell invasion. Mol Cancer Res *6*, 1544-1553.
- Moore BW (1965). A soluble protein characteristic of the nervous system. Biochem and Biophys Res Comms *19*, 739-744.
- Moore, M. J., Goldstein, D., Hamm, J., Figer, A., Hecht, J. R., Gallinger, S., Au, H. J., Murawa, P., Walde, D., Wolff, R. A., *et al.* (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol *25*, 1960-1966.
- Moore, P. S., Sipos, B., Orlandini, S., Sorio, C., Real, F. X., Lemoine, N. R., Gress, T., Bassi, C., Kloppel, G., Kalthoff, H., *et al.* (2001). Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. Virchows Arch *439*, 798-802.
- Moore R, Carlson S, and Madara JL (1998). Villus contraction aids repair ofintestinal epithelium after injury. Am J Physiol *257*, G274-G283.
- Motoi, F., Sunamura, M., Ding, L., Duda, D. G., Yoshida, Y., Zhang, W., Matsuno, S., and Hamada, H. (2000). Effective gene therapy for pancreatic cancer by cytokines mediated by restricted replication-competent adenovirus. Hum Gene Ther *11*, 223-235.
- Mueller, A., Schafer, B. W., Ferrari, S., Weibel, M., Makek, M., Hochli, M., and Heizmann, C. W. (2005). The calcium-binding protein S100A2 interacts with p53 and modulates its transcriptional activity. J Biol Chem *280*, 29186-29193.
- Muller, N., Reinacher-Schick, A., Baldus, S., van Hengel, J., Berx, G., Baar, A., van Roy, F., Schmiegel, W., and Schwarte-Waldhoff, I. (2002). Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells. Oncogene *21*, 6049-6058.
- Munshi, H. G., and Stack, M. S. (2006). Reciprocal interactions between adhesion receptor signaling and MMP regulation. Cancer Metastasis Rev *25*, 45-56.
- Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer *8*, 618-631.
- Nacken, W., Roth, J., Sorg, C., and Kerkhoff, C. (2003). S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech *60*, 569-580.
- Nedjadi, T., Kitteringham, N., Campbell, F., Jenkins, R. E., Park, B. K., Navarro, P., Ashcroft, F., Tepikin, A., Neoptolemos, J. P., and Costello, E. (2009). S100A6 binds to annexin 2 in pancreatic cancer cells and promotes pancreatic cancer cell motility. Br J Cancer *101*, 1145-1154.
- Neel, N. F., Martin, T. D., Stratford, J. K., Zand, T. P., Reiner, D. J., and Der, C. J. (2011). The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. Genes Cancer *2*, 275-287.
- Neesse, A., Michl, P., Frese, K. K., Feig, C., Cook, N., Jacobetz, M. A., Lolkema, M. P., Buchholz, M., Olive, K. P., Gress, T. M., and Tuveson, D. A. (2011). Stromal biology and therapy in pancreatic cancer. Gut *60*, 861-868.

- Nemeth, J., Stein, I., Haag, D., Riehl, A., Longerich, T., Horwitz, E., Breuhahn, K., Gebhardt, C., Schirmacher, P., Hahn, M., *et al.* (2009). S100A8 and S100A9 are novel nuclear factor kappa B target genes during malignant progression of murine and human liver carcinogenesis. Hepatology *50*, 1251-1262.
- Neoptolemos, J. P. (2011). Adjuvant treatment of pancreatic cancer. Eur J Cancer *47 Suppl 3*, S378-380.
- Neoptolemos, J. P., Stocken, D. D., Friess, H., Bassi, C., Dunn, J. A., Hickey, H., Beger, H., Fernandez-Cruz, L., Dervenis, C., Lacaine, F., *et al.* (2004). A Randomized Trial of Chemoradiotherapy and Chemotherapy after Resection of Pancreatic Cancer
- 10.1056/NEJMoa032295. N Engl J Med *350*, 1200-1210.
- Neoptolemos, J. P., Stocken, D. D., Tudur Smith, C., Bassi, C., Ghaneh, P., Owen, E., Moore, M., Padbury, R., Doi, R., Smith, D., and Buchler, M. W. (2009). Adjuvant 5-fluorouracil and folinic acid vs observation for pancreatic cancer: composite data from the ESPAC-1 and -3(v1) trials. Br J Cancer *100*, 246-250.
- Nesbit, M., Schaider, H., Miller, T. H., and Herlyn, M. (2001). Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells. J Immunol *166*, 6483-6490.
- Nguyen DHD, Webb DJ, Catling AD, Song Q, Dhakephalkar A, Weber J, Ravichandran KS, and Gonias SL (2000). Urokinase-type plasminogen activator stimulates the ras/extracellular signal-regulated kinase (ERK) signalling pathway and MCF-7 cell migration by a mechanism that requires focal adhesion kinase, src, and shc: rapid dissociation of grb2/sos-shc complex is associated with the transient phosphorylation of ERK in urokinase-treated cells. J Biol Chem *275*, 19382-19388.
- Nicholas FJ, and Hills CS (2003). Attenuation of the TGF-beta-Smad pathway in pancreatic tumour cells confers resistance to TGF-beta induced growth arrest. Oncogene *22*, 3698-3711.
- Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., and et al. (1989). Mutations in the p53 gene occur in diverse human tumour types. Nature *342*, 705-708.
- Noone PG, Zhou Z, Silverman LM, Jowell PS, Knowles MR, and Cohn JA (2001). Cystic fibrosis gene mutations and pancreatitis risk: relation to epithelial ion transport and trypsin inhibitor gene mutations. Gastroenterology *121*, 1310-1319.
- O'Hagan, R. C., Chang, S., Maser, R. S., Mohan, R., Artandi, S. E., Chin, L., and DePinho, R. A. (2002). Telomere dysfunction provokes regional amplification and deletion in cancer genomes. Cancer Cell *2*, 149-155.
- Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987). Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature *330*, 80-82.
- Offerhaus, G. J., Giardiello, F. M., Krush, A. J., Booker, S. V., Tersmette, A. C., Kelley, N. C., and Hamilton, S. R. (1992). The risk of upper gastrointestinal cancer in familial adenomatous polyposis. Gastroenterology *102*, 1980-1982.

- Ohuchida, K., Mizumoto, K., Ishikawa, N., Fujii, K., Konomi, H., Nagai, E., Yamaguchi, K., Tsuneyoshi, M., and Tanaka, M. (2005). The Role of S100A6 in Pancreatic Cancer Development and Its Clinical Implication as a Diagnostic Marker and Therapeutic Target
- 10.1158/1078-0432.CCR-05-0714. Clin Cancer Res *11*, 7785-7793.
- Ohuchida, K., Mizumoto, K., Miyasaka, Y., Yu, J., Cui, L., Yamaguchi, H., Toma, H., Takahata, S., Sato, N., Nagai, E., *et al.* (2007a). Over-expression of S100A2 in pancreatic cancer correlates with progression and poor prognosis. J Pathol *213*, 275-282.
- Ohuchida, K., Mizumoto, K., Ohhashi, S., Yamaguchi, H., Konomi, H., Nagai, E., Yamaguchi, K., Tsuneyoshi, M., and Tanaka, M. (2006). S100A11, a putative tumor suppressor gene, is overexpressed in pancreatic carcinogenesis. Clin Cancer Res *12*, 5417-5422.
- Ohuchida, K., Mizumoto, K., Yu, J., Yamaguchi, H., Konomi, H., Nagai, E., Yamaguchi, K., Tsuneyoshi, M., and Tanaka, M. (2007b). S100A6 is increased in a stepwise manner during pancreatic carcinogenesis: clinical value of expression analysis in 98 pancreatic juice samples. Cancer Epidemiol Biomarkers Prev *16*, 649-654.
- Olive, K. P., Jacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., Madhu, B., Goldgraben, M. A., Caldwell, M. E., Allard, D., *et al.* (2009). Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science *324*, 1457-1461.
- Olson, S. H. (2011). Selected medical conditions and risk of pancreatic cancer. Mol Carcinog *51*, 75-97.
- Ott, H. W., Lindner, H., Sarg, B., Mueller-Holzner, E., Abendstein, B., Bergant, A., Fessler, S., Schwaerzler, P., Zeimet, A., Marth, C., and Illmensee, K. (2003). Calgranulins in cystic fluid and serum from patients with ovarian carcinomas. Cancer Res *63*, 7507-7514.
- Ozawa, F., Friess, H., Tempia-Caliera, A., Kleeff, J., and Buchler, M. W. (2001). Growth factors and their receptors in pancreatic cancer. Teratog Carcinog Mutagen *21*, 27-44.
- Ozcelik H, Scmocker B, Di Nicola N, and et al. (1997). Germline BRACA2 6174delT mutations in Ashkenazi Jewish cancer patients [Letter]. Nat Genet *16*, 17-18.
- Paliwal, S., Pande, S., Kovi, R. C., Sharpless, N. E., Bardeesy, N., and Grossman, S. R. (2006). Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. Mol Cell Biol *26*, 2360-2372.
- Pandey, A., Forte, V., Abdallah, M., Alickaj, A., Mahmud, S., Asad, S., and McFarlane, S. I. (2011). Diabetes mellitus and the risk of cancer. Minerva endocrinologica *36*, 187-209.
- Pandol, S., Edderkaoui, M., Gukovsky, I., Lugea, A., and Gukovskaya, A. (2009). Desmoplasia of pancreatic ductal adenocarcinoma. Clin Gastroenterol Hepatol *7*, S44-47.
- Peltomaki, P., and Vasen, H. (2004). Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/INSiGHT mutation database. Dis Markers *20*, 269-276.

- Phillips PA, McCarroll JA, Park S, Wu MJ, Pirola R, Korsten M, Wilson JS, and Apte MV (2003). Rat pancreatic stellate cells secrete matrix metalloproteinases: implications for extracellular matrix turnover. Gut *52*, 275-282.
- Phillips, P. A., Wu, M. J., Kumar, R. K., Doherty, E., McCarroll, J. A., Park, S., Pirola, R. C., Wilson, J. S., and Apte, M. V. (2003). Cell migration: a novel aspect of pancreatic stellate cell biology. Gut *52*, 677-682.
- Pietenpol, J. A., Holt, J. T., Stein, R. W., and Moses, H. L. (1990). Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci U S A *87*, 3758-3762.
- Plentz, R., Park, J. S., Rhim, A. D., Abravanel, D., Hezel, A. F., Sharma, S. V., Gurumurthy, S., Deshpande, V., Kenific, C., Settleman, J., *et al.* (2009). Inhibition of gamma-secretase activity inhibits tumor progression in a mouse model of pancreatic ductal adenocarcinoma. Gastroenterology *136*, 1741-1749 e1746.
- Pogue-Geile, K. L., Chen, R., Bronner, M. P., Crnogorac-Jurcevic, T., Moyes, K. W., Dowen, S., Otey, C. A., Crispin, D. A., George, R. D., Whitcomb, D. C., and Brentnall, T. A. (2006). Palladin mutation causes familial pancreatic cancer and suggests a new cancer mechanism. PLoS Med *3*, e516.
- Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer *4*, 71-78.
- Polyak, K., Haviv, I., and Campbell, I. G. (2009). Co-evolution of tumor cells and their microenvironment. Trends Genet *25*, 30-38.
- Porta, M., Crous-Bou, M., Wark, P. A., Vineis, P., Real, F. X., Malats, N., and Kampman, E. (2009). Cigarette smoking and K-ras mutations in pancreas, lung and colorectal adenocarcinomas: etiopathogenic similarities, differences and paradoxes. Mutat Res *682*, 83-93.
- Prokopchuk, O., Liu, Y., Henne-Bruns, D., and Kornmann, M. (2005). Interleukin-4 enhances proliferation of human pancreatic cancer cells: evidence for autocrine and paracrine actions. Br J Cancer *92*, 921-928.
- Pupa SM, Menard S, Forti S, and Tagliabue E (2002). New insights into the role of extracellular matrix during tumour onset and progression. J Cell Physiol *192*, 259-267.
- Qian, B. Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., Kaiser, E. A., Snyder, L. A., and Pollard, J. W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature *475*, 222-225.
- Rafii, S., and Lyden, D. (2006). S100 chemokines mediate bookmarking of premetastatic niches. Nat Cell Biol *8*, 1321-1323.
- Rahimi, F., Hsu, K., Endoh, Y., and Geczy, C. L. (2005). FGF-2, IL-1beta and TGF-beta regulate fibroblast expression of S100A8. Febs J *272*, 2811-2827.
- Raimondi, S., Maisonneuve, P., Lohr, J. M., and Lowenfels, A. B. (2007). Early onset pancreatic cancer: evidence of a major role for smoking and genetic factors. Cancer Epidemiol Biomarkers Prev *16*, 1894-1897.
- Raimondi, S., Maisonneuve, P., and Lowenfels, A. B. (2009). Epidemiology of pancreatic cancer: an overview. Nat Rev Gastroenterol Hepatol *6*, 699-708.

- Rammes, A., Roth, J., Goebeler, M., Klempt, M., Hartmann, M., and Sorg, C. (1997). Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem *272*, 9496-9502.
- Ranganathan, P., Weaver, K. L., and Capobianco, A. J. (2011). Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer *11*, 338-351.
- Raulet, D. H., and Guerra, N. (2009). Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol *9*, 568-580.
- Redston MS, Caldas C, Seymour, A., Hruban RH, da Costa L T, Yeo CJ, and Kern SE (1994). p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. Canc Res *54*, 3025-3033.
- Rehman, A. O., and Wang, C. Y. (2006). Notch signaling in the regulation of tumor angiogenesis. Trends Cell Biol *16*, 293-300.
- Rhim, A. D., Mirek, E. T., Aiello, N. M., Maitra, A., Bailey, J. M., McAllister, F., Reichert, M., Beatty, G. L., Rustgi, A. K., Vonderheide, R. H., *et al.* (2012). EMT and dissemination precede pancreatic tumor formation. Cell *148*, 349-361.
- Ribatti, D., Nico, B., Crivellato, E., Roccaro, A. M., and Vacca, A. (2007). The history of the angiogenic switch concept. Leukemia *21*, 44-52.
- Richards, N. G., Rittenhouse, D. W., Freydin, B., Cozzitorto, J. A., Grenda, D., Rui, H., Gonye, G., Kennedy, E. P., Yeo, C. J., Brody, J. R., and Witkiewicz, A. K. (2010). HuR status is a powerful marker for prognosis and response to gemcitabine-based chemotherapy for resected pancreatic ductal adenocarcinoma patients. Ann Surg *252*, 499-505; discussion 505-496.
- Robinson, S. C., Scott, K. A., and Balkwill, F. R. (2002). Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. Eur J Immunol *32*, 404-412.
- Rocha-Lima, C. M. (2008). New directions in the management of advanced pancreatic cancer: a review. Anticancer Drugs *19*, 435-446.
- Rocha, S., Campbell, K. J., and Perkins, N. D. (2003). p53- and Mdm2-independent repression of NF-kappa B transactivation by the ARF tumor suppressor. Mol Cells *12*, 15-25.
- Rodenhuis, S. (1992). ras and human tumors. Semin Cancer Biol *3*, 241-247.
- Rodrigues, N. R., Rowan, A., Smith, M. E., Kerr, I. B., Bodmer, W. F., Gannon, J. V., and Lane, D. P. (1990). p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A *87*, 7555-7559.
- Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996). Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. Embo J *15*, 2442-2451.
- Rohrmann, S., Linseisen, J., Vrieling, A., Boffetta, P., Stolzenberg-Solomon, R. Z., Lowenfels, A. B., Jensen, M. K., Overvad, K., Olsen, A., Tjonneland, A., *et al.* (2009). Ethanol intake and the risk of pancreatic cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC). Cancer Causes Control *20*, 785-794.

- Rooney PH, Murray GI, Stevenson DAJ, Haites NE, Cassidy J, and McLeoud HL (1999). Comaparative genomic hybidisation and chromosomal instability in solid tumours. Br J Cancer *80*, 862-873.
- Rosty, C., Ueki, T., Argani, P., Jansen, M., Yeo, C. J., Cameron, J. L., Hruban, R. H., and Goggins, M. (2002). Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. Am J Pathol *160*, 45-50.
- Roth J, Burwinkel F, van den Bos C, Goebeler M, Vollmer E, and Sorg C (1993). MRP8 and MRP14, S100 like proteins associated with myeloid differentiation, are translocated to plasma membranes and intermediate filaments in a calcium dependant manner. Blood *82*, 1875-1883.
- Roth, J., Goebeler, M., and Sorg, C. (2001). S100A8 and S100A9 in inflammatory diseases. Lancet *357*, 1041.
- Roth, J., Vogl, T., Sorg, C., and Sunderkotter, C. (2003). Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends Immunol *24*, 155-158.
- Rozenblum, E., Schutte, M., Goggins, M., Hahn, S. A., Panzer, S., Zahurak, M., Goodman, S. N., Sohn, T. A., Hruban, R. H., Yeo, C. J., and Kern, S. E. (1997). Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res *57*, 1731-1734.
- Rudland, P. S., Platt-Higgins, A., Renshaw, C., West, C. R., Winstanley, J. H., Robertson, L., and Barraclough, R. (2000). Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. Cancer Res *60*, 1595-1603.
- Ruffell, B., Affara, N. I., and Coussens, L. M. (2012). Differential macrophage programming in the tumor microenvironment. Trends Immunol *33*, 119-126.
- Ruijs, M. W., Verhoef, S., Rookus, M. A., Pruntel, R., van der Hout, A. H., Hogervorst, F. B., Kluijt, I., Sijmons, R. H., Aalfs, C. M., Wagner, A., *et al.* (2010). TP53 germline mutation testing in 180 families suspected of Li-Fraumeni syndrome: mutation detection rate and relative frequency of cancers in different familial phenotypes. J Med Genet *47*, 421-428.
- Ruoslahti E (2002). Specialisation of tumour vasculature. Nature Reviews *2*, 83-90.
- Ryckman, C., Gilbert, C., de Medicis, R., Lussier, A., Vandal, K., and Tessier, P. A. (2004). Monosodium urate monohydrate crystals induce the release of the proinflammatory protein S100A8/A9 from neutrophils. J Leukoc Biol *76*, 433-440.
- Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., and Tessier, P. A. (2003). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol *170*, 3233-3242.
- Salama, I., Malone, P. S., Mihaimeed, F., and Jones, J. L. (2008). A review of the S100 proteins in cancer. Eur J Surg Oncol *34*, 357-364.
- Santamaria-Kisiel, L., Rintala-Dempsey, A. C., and Shaw, G. S. (2006). Calcium-dependent and -independent interactions of the S100 protein family. Biochem J *396*, 201-214.
- Saruc M, and Pour PM (2003). Diabetes and its relationship to pancreatic carcinoma. Pancreas *26*, 381-387.

- Sato, N., Mizumoto, K., Nagai, E., and Tanaka, M. (2002). Telomerase as a new target for pancreatic cancer treatment. J Hepatobiliary Pancreat Surg *9*, 322-327.
- Scarlett, C. J., Salisbury, E. L., Biankin, A. V., and Kench, J. (2011). Precursor lesions in pancreatic cancer: morphological and molecular pathology. Pathology *43*, 183-200.
- Schafer BW, and Heizmann CW (1996). The S100 family of EF-hand calcium binding proteins: functions and pathology. Trends Biochem Sci *21*, 134-140.
- Schernhammer ES, Michaud DS, Leitzmann MF, Giovannucci E, Colditz GA, and Fuchs CS (2002). Gallstones, cholecystectomy, and the risk of devloping pancreatic cancer. Br J Cancer *86*, 1081-1084.
- Schlueter, C., Weber, H., Meyer, B., Rogalla, P., Roser, K., Hauke, S., and Bullerdiek, J. (2005). Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule. Am J Pathol *166*, 1259-1263.
- Schmid, M. C., and Varner, J. A. (2007). Myeloid cell trafficking and tumor angiogenesis. Cancer Lett *250*, 1-8.
- Schmiegel, W., Roeder, C., Schmielau, J., Rodeck, U., and Kalthoff, H. (1993). Tumor necrosis factor alpha induces the expression of transforming growth factor alpha and the epidermal growth factor receptor in human pancreatic cancer cells. Proc Natl Acad Sci U S A *90*, 863-867.
- Schmierer, B., and Hill, C. S. (2007). TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol *8*, 970-982.
- Schmitz-Winnenthal, F. H., Volk, C., Z'Graggen, K., Galindo, L., Nummer, D., Ziouta, Y., Bucur, M., Weitz, J., Schirrmacher, V., Buchler, M. W., and Beckhove, P. (2005). High frequencies of functional tumor-reactive T cells in bone marrow and blood of pancreatic cancer patients. Cancer Res *65*, 10079-10087.
- Schonherr, E., Levkau, B., Schaefer, L., Kresse, H., and Walsh, K. (2001). Decorin-mediated signal transduction in endothelial cells. Involvement of Akt/protein kinase B in up-regulation of p21(WAF1/CIP1) but not p27(KIP1). J Biol Chem *276*, 40687-40692.
- Schoppmann, S. F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K., and Kerjaschki, D. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol *161*, 947-956.
- Schoumacher, R. A., Ram, J., Iannuzzi, M. C., Bradbury, N. A., Wallace, R. W., Hon, C. T., Kelly, D. R., Schmid, S. M., Gelder, F. B., Rado, T. A., and et al. (1990). A cystic fibrosis pancreatic adenocarcinoma cell line. Proc Natl Acad Sci U S A *87*, 4012-4016.
- Schuller HM (2002). Mechanisms of smoking related lung and pancreatic adenocarcinoma devlopment. Nat Rev Cancer *2*, 455-463.
- Schwarte-Waldhoff, I., Klein, S., Blass-Kampmann, S., Hintelmann, A., Eilert, C., Dreschers, S., Kalthoff, H., Hahn, S. A., and Schmiegel, W. (1999). DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression. Oncogene *18*, 3152-3158.

- Schwarte-Waldhoff, I., and Schmiegel, W. (2002). Smad4 transcriptional pathways and angiogenesis. Int J Gastrointest Cancer *31*, 47-59.
- Schwarte-Waldhoff, I., Volpert, O. V., Bouck, N. P., Sipos, B., Hahn, S. A., Klein-Scory, S., Luttges, J., Kloppel, G., Graeven, U., Eilert-Micus, C., *et al.* (2000). Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc Natl Acad Sci U S A *97*, 9624-9629.
- Scotto C, Deloulme JC, Rousseau D, Chambaz E, and Baudier J (1998). Calcium and S100B regulation of p53 dependant cell growth arrest and apoptosis. Mol Cell Biol *18*, 4272-4281.
- Scotton, C., Milliken, D., Wilson, J., Raju, S., and Balkwill, F. (2001). Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. Br J Cancer *85*, 891-897.
- Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. Faseb J *9*, 726-735.
- Seo, Y., Baba, H., Fukuda, T., Takashima, M., and Sugimachi, K. (2000). High expression of vascular endothelial growth factor is associated with liver metastasis and a poor prognosis for patients with ductal pancreatic adenocarcinoma. Cancer *88*, 2239-2245.
- Seymour, A. B., Hruban, R. H., Redston, M., Caldas, C., Powell, S. M., Kinzler, K. W., Yeo, C. J., and Kern, S. E. (1994). Allelotype of pancreatic adenocarcinoma. Cancer Res *54*, 2761-2764.
- Shannon AM, Bouchier-Hayes DJ, Condron CM, and Toomey D (2003). Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treatment Reviews *29*, 297-307.
- Shapiro, I. M., Cheng, A. W., Flytzanis, N. C., Balsamo, M., Condeelis, J. S., Oktay, M. H., Burge, C. B., and Gertler, F. B. (2011). An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype. PLoS Genet *7*, e1002218.
- Sheikh, A. A., Vimalachandran, D., Thompson, C. C., Jenkins, R. E., Nedjadi, T., Shekouh, A., Campbell, F., Dodson, A., Prime, W., Crnogorac-Jurcevic, T., *et al.* (2007). The expression of S100A8 in pancreatic cancer-associated monocytes is associated with the Smad4 status of pancreatic cancer cells. Proteomics *7*, 1929-1940.
- Sherr, C. J. (2004). Principles of tumor suppression. Cell *116*, 235-246.
- Sherr, C. J. (2006). Divorcing ARF and p53: an unsettled case. Nat Rev Cancer *6*, 663-673.
- Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.
- Shibata, F., Miyama, K., Shinoda, F., Mizumoto, J., Takano, K., and Nakagawa, H. (2004). Fibroblast growth-stimulating activity of S100A9 (MRP-14). Eur J Biochem *271*, 2137-2143.
- Shields, M. A., Dangi-Garimella, S., Krantz, S. B., Bentrem, D. J., and Munshi, H. G. (2012a). Pancreatic cancer cells respond to type I collagen by inducing snail expression to promote membrane type 1 matrix metalloproteinase-dependent collagen invasion. J Biol Chem *286*, 10495-10504.

- Shields, M. A., Dangi-Garimella, S., Redig, A. J., and Munshi, H. G. (2012b). Biochemical role of the collagen-rich tumour microenvironment in pancreatic cancer progression. Biochem J *441*, 541-552.
- Sica, A., Schioppa, T., Mantovani, A., and Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer *42*, 717-727.
- Siegel, R., Naishadham, D., and Jemal, A. (2012). Cancer statistics, 2012. CA Cancer J Clin *62*, 10-29.
- Sieweke, M. H., and Bissell, M. J. (1994). The tumor-promoting effect of wounding: a possible role for TGF-beta-induced stromal alterations. Crit Rev Oncog *5*, 297-311.
- Silverman DT, Hoover RN, Brown LM, Swanson GM, Schiffman M, Greenburg RS, Hayes RB, Lillemore KD, Schoenberg JB, Schwartz AG, *et al.* (2003). Why do black Americans have a higher risk of pancreatic cancer than white Americans ? Epidemiology *14(1)*, 45-54.
- Simon-Assmann P, Kedinger M, de Arcangelis A, Rosseau V, and Simo P (1995). Extracellular matrix components in intestinal devlopment. Experientia *51*, 883-900.
- Sims, J. E., March, C. J., Cosman, D., Widmer, M. B., MacDonald, H. R., McMahan, C. J., Grubin, C. E., Wignall, J. M., Jackson, J. L., Call, S. M., and et al. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science *241*, 585-589.
- Singh, P., Wig, J. D., and Srinivasan, R. (2011). The Smad family and its role in pancreatic cancer. Indian J Cancer *48*, 351-360.
- Sipos, B., Moser, S., Kalthoff, H., Torok, V., Lohr, M., and Kloppel, G. (2003). A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. Virchows Arch *442*, 444-452.
- Sipos, F., and Galamb, O. (2012). Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. World J Gastroenterol *18*, 601-608.
- Siveen, K. S., and Kuttan, G. (2009). Role of macrophages in tumour progression. Immunol Lett *123*, 97-102.
- Skinner HG, Michaud DS, Giovannucci EL, Rimm EB, Stampfer MJ, Willett WC, Colditz GA, and Fuchs CS (2004). A prospective study of folate intake and the risk of pancreatic cancer in men and women. Am J Epidemiol *160*, 248-258.
- Smith, S. L., Gugger, M., Hoban, P., Ratschiller, D., Watson, S. G., Field, J. K., Betticher, D. C., and Heighway, J. (2004). S100A2 is strongly expressed in airway basal cells, preneoplastic bronchial lesions and primary non-small cell lung carcinomas. Br J Cancer *91*, 1515-1524.
- Society, A. C. (2010). Estimated New Cancer Cases and Deaths by Sex, US, 2009. http://wwwcancerorg/docroot/MED/content/MED_1_1_Most_Requested_Graphs_and_Figures_2009asp.
- Solinas, G., Germano, G., Mantovani, A., and Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol *86*, 1065-1073.

- Song, S., Ewald, A. J., Stallcup, W., Werb, Z., and Bergers, G. (2005). PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat Cell Biol *7*, 870-879.
- Spano, J. P., Chodkiewicz, C., Maurel, J., Wong, R., Wasan, H., Barone, C., Letourneau, R., Bajetta, E., Pithavala, Y., Bycott, P., *et al.* (2008). Efficacy of gemcitabine plus axitinib compared with gemcitabine alone in patients with advanced pancreatic cancer: an open-label randomised phase II study. Lancet *371*, 2101-2108.
- Srikrishna, G. (2012). S100A8 and S100A9: new insights into their roles in malignancy. J Innate Immun *4*, 31-40.
- Stamenkovic I (2003). Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol *200*, 448-464.
- Strieter, R. M., Belperio, J. A., Phillips, R. J., and Keane, M. P. (2004). CXC chemokines in angiogenesis of cancer. Semin Cancer Biol *14*, 195-200.
- Striz, I., and Trebichavsky, I. (2004). Calprotectin - a pleiotropic molecule in acute and chronic inflammation. Physiol Res *53*, 245-253.
- Strouch, M. J., Cheon, E. C., Salabat, M. R., Krantz, S. B., Gounaris, E., Melstrom, L. G., Dangi-Garimella, S., Wang, E., Munshi, H. G., Khazaie, K., and Bentrem, D. J. (2010). Crosstalk between mast cells and pancreatic cancer cells contributes to pancreatic tumor progression. Clin Cancer Res *16*, 2257-2265.
- Stuhler, K., Koper, K., Pfeiffer, K., Tagariello, A., Souquet, M., Schwarte-Waldhoff, I., Hahn, S. A., Schmiegel, W., and Meyer, H. E. (2006). Differential proteome analysis of colon carcinoma cell line SW480 after reconstitution of the tumour suppressor Smad4. Anal Bioanal Chem *386*, 1603-1612.
- Stulik J, Osterreicher J, Koupilova K, Knizek J, Macela A, Bures J, Jandik P, Langr F, Dedic K, and Jungblut PR (1999). The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: The S100A9 and S100A8 positive cells underlie and invade tumour mass. Electrophoresis *20*, 1047-1054.
- Stulik, J., Osterreicher, J., Koupilova, K., Knizek, J., Bures, J., Jandik, P., Langr, F., Dedic, K., Schafer, B. W., and Heizmann, C. W. (2000). Differential expression of the Ca2+ binding S100A6 protein in normal, preneoplastic and neoplastic colon mucosa. Eur J Cancer *36*, 1050-1059.
- Sundaram, M. V. (2005). The love-hate relationship between Ras and Notch. Genes Dev *19*, 1825-1839.
- Suryono, Kido, J., Hayashi, N., Kataoka, M., Shinohara, Y., and Nagata, T. (2006). Norepinephrine stimulates calprotectin expression in human monocytic cells. J Periodontal Res *41*, 159-164.
- Tabata, T., Tsukamoto, N., Fooladi, A. A., Yamanaka, S., Furukawa, T., Ishida, M., Sato, D., Gu, Z., Nagase, H., Egawa, S., *et al.* (2009). RNA interference targeting against S100A4 suppresses cell growth and motility and induces apoptosis in human pancreatic cancer cells. Biochem Biophys Res Commun *390*, 475-480.
- Takaya, H., Andoh, A., Shimada, M., Hata, K., Fujiyama, Y., and Bamba, T. (2000). The expression of chemokine genes correlates with nuclear factor-kappaB activation in human pancreatic cancer cell lines. Pancreas *21*, 32-40.

- Tan, M. H., Nowak, N. J., Loor, R., Ochi, H., Sandberg, A. A., Lopez, C., Pickren, J. W., Berjian, R., Douglass, H. O., Jr., and Chu, T. M. (1986). Characterization of a new primary human pancreatic tumor line. Cancer Invest *4*, 15-23.
- Tartour, E., Pere, H., Maillere, B., Terme, M., Merillon, N., Taieb, J., Sandoval, F., Quintin-Colonna, F., Lacerda, K., Karadimou, A., *et al.* (2011
-). Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. Cancer Metastasis Rev *30*, 83-95.
- Tascilar M, van Rees BP, Sturm PDJ, Tytgat GN, Hruban RH, Goodman SN, Giardiello FM, Offerhaus GJA, and Tersmette AC (2002). Pancreatic cancer after remote peptic ulcer surgery. J Clin Pathol *55*, 340-345.
- Tascilar, M., Skinner, H. G., Rosty, C., Sohn, T., Wilentz, R. E., Offerhaus, G. J., Adsay, V., Abrams, R. A., Cameron, J. L., Kern, S. E., *et al.* (2001). The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin Cancer Res *7*, 4115-4121.
- Thiel C, Osborn M, and Gerke V (1992). The tight association of the tyrosine kinase substrate annexin II with the submembranous cytoskeleton depends on intact p11 and Ca^{2+} binding sites. J Cell Biol *103*, 733-742.
- Thomas, A., Dajani, K., Neoptolemos, J. P., and Ghaneh, P. (2010
-). Adjuvant therapy in pancreatic cancer. Dig Dis *28*, 684-692.
- Thompson, C. C., Ashcroft, F. J., Patel, S., Saraga, G., Vimalachandran, D., Prime, W., Campbell, F., Dodson, A., Jenkins, R. E., Lemoine, N. R., *et al.* (2007). Pancreatic cancer cells overexpress gelsolin family-capping proteins, which contribute to their cell motility. GUT *56*, 95-106.
- Thompson D, and Easton DF (2002). Cancer incidence in BRCA1 mutation carriers. J Natl Canc Inst *94*, 409-416.
- Tibble, J., Teahon, K., Thjodleifsson, B., Roseth, A., Sigthorsson, G., Bridger, S., Foster, R., Sherwood, R., Fagerhol, M., and Bjarnason, I. (2000). A simple method for assessing intestinal inflammation in Crohn's disease. Gut *47*, 506-513.
- Tjomsland, V., Niklasson, L., Sandstrom, P., Borch, K., Druid, H., Bratthall, C., Messmer, D., Larsson, M., and Spangeus, A. (2011). The desmoplastic stroma plays an essential role in the accumulation and modulation of infiltrated immune cells in pancreatic adenocarcinoma. Clin Dev Immunol *2011*, 212810.
- Trainer, D. L., Kline, T., McCabe, F. L., Faucette, L. F., Feild, J., Chaikin, M., Anzano, M., Rieman, D., Hoffstein, S., Li, D. J., and et al. (1988). Biological characterization and oncogene expression in human colorectal carcinoma cell lines. Int J Cancer *41*, 287-296.
- Trikudanathan, G., Philip, A., Dasanu, C. A., and Baker, W. L. (2012). Association between Helicobacter pylori infection and pancreatic cancer. A cumulative meta-analysis. Jop *12*, 26-31.
- Troiani, T., Martinelli, E., Capasso, A., Morgillo, F., Orditura, M., De Vita, F., and Ciardiello, F. (2012). Targeting EGFR in Pancreatic Cancer Treatment. Curr Drug Targets.

- Truty, M. J., and Urrutia, R. (2007a). Basics of TGF-beta and pancreatic cancer. Pancreatology *7*, 423-435.
- Truty, M. J., and Urrutia, R. (2007b). Transforming growth factor-beta: what every pancreatic surgeon should know. Surgery *141*, 1-6.
- Tuveson, D. A., and Neoptolemos, J. P. (2012). Understanding metastasis in pancreatic cancer: a call for new clinical approaches. Cell *148*, 21-23.
- Valenti, R., Huber, V., Filipazzi, P., Pilla, L., Sovena, G., Villa, A., Corbelli, A., Fais, S., Parmiani, G., and Rivoltini, L. (2006). Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. Cancer Res *66*, 9290-9298.
- Valkovic, T., Dobrila, F., Melato, M., Sasso, F., Rizzardi, C., and Jonjic, N. (2002). Correlation between vascular endothelial growth factor, angiogenesis, and tumor-associated macrophages in invasive ductal breast carcinoma. Virchows Arch *440*, 583-588.
- Van Cutsem, E., Vervenne, W. L., Bennouna, J., Humblet, Y., Gill, S., Van Laethem, J. L., Verslype, C., Scheithauer, W., Shang, A., Cosaert, J., and Moore, M. J. (2009). Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. J Clin Oncol *27*, 2231-2237.
- van der Heijden, M. S., Brody, J. R., Gallmeier, E., Cunningham, S. C., Dezentje, D. A., Shen, D., Hruban, R. H., and Kern, S. E. (2004). Functional defects in the fanconi anemia pathway in pancreatic cancer cells. Am J Pathol *165*, 651-657.
- van Heek NT, Meeker AK, Kern S, Yeo CJ, Lillemore KD, Cameron JL, Offerhaus GJA, Hicks JL, Wilentz RE, Goggins M, *et al.* (2002). Telomere shortening is nearly universal in pancreatic intraepithelial neoplasisa. Am J Pathol *161*, 1541-1547.
- Van Hoorde, L., Van Aken, E., and Mareel, M. (2000). Collagen type I: a substrate and a signal for invasion. Prog Mol Subcell Biol *25*, 105-134.
- Vasen, H. F., Gruis, N. A., Frants, R. R., van Der Velden, P. A., Hille, E. T., and Bergman, W. (2000). Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). Int J Cancer *87*, 809-811.
- Vimalachandran, D., Greenhalf, W., Thompson, C., Luttges, J., Prime, W., Campbell, F., Dodson, A., Watson, R., Crnogorac-Jurcevic, T., Lemoine, N., *et al.* (2005). High nuclear S100A6 (Calcyclin) is significantly associated with poor survival in pancreatic cancer patients. Cancer Res *65*, 3218-3225.
- Vinay Kumar, A. K. A., Nelson Fausto , Richard Mitchell (2007). Robbins Basic Pathology. Saunders *8 edition*.
- Vincent, A., Herman, J., Schulick, R., Hruban, R. H., and Goggins, M. (2011). Pancreatic cancer. Lancet *378*, 607-620.
- Vitone, L. J., Greenhalf, W., McFaul, C. D., Ghaneh, P., and Neoptolemos, J. P. (2006). The inherited genetics of pancreatic cancer and prospects for secondary screening. Best Pract Res Clin Gastroenterol *20*, 253-283.
- Vogl, T., Ludwig, S., Goebeler, M., Strey, A., Thorey, I. S., Reichelt, R., Foell, D., Gerke, V., Manitz, M. P., Nacken, W., *et al.* (2004). MRP8 and MRP14

control microtubule reorganization during transendothelial migration of phagocytes. Blood *104*, 4260-4268.

- Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M. A., Nacken, W., Foell, D., van der Poll, T., Sorg, C., and Roth, J. (2007). Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med *13*, 1042-1049.
- Volmer, M. W., Radacz, Y., Hahn, S. A., Klein-Scory, S., Stuhler, K., Zapatka, M., Schmiegel, W., Meyer, H. E., and Schwarte-Waldhoff, I. (2004). Tumor suppressor Smad4 mediates downregulation of the anti-adhesive invasion-promoting matricellular protein SPARC: Landscaping activity of Smad4 as revealed by a "secretome" analysis. Proteomics *4*, 1324-1334.
- Volmer, M. W., Stuhler, K., Zapatka, M., Schoneck, A., Klein-Scory, S., Schmiegel, W., Meyer, H. E., and Schwarte-Waldhoff, I. (2005). Differential proteome analysis of conditioned media to detect Smad4 regulated secreted biomarkers in colon cancer. Proteomics *5*, 2587-2601.
- Vonlaufen, A., Joshi, S., Qu, C., Phillips, P. A., Xu, Z., Parker, N. R., Toi, C. S., Pirola, R. C., Wilson, J. S., Goldstein, D., and Apte, M. V. (2008). Pancreatic stellate cells: partners in crime with pancreatic cancer cells. Cancer Res *68*, 2085-2093.
- Vrieling, A., Verhage, B. A., van Duijnhoven, F. J., Jenab, M., Overvad, K., Tjonneland, A., Olsen, A., Clavel-Chapelon, F., Boutron-Ruault, M. C., Kaaks, R., *et al.* (2009). Fruit and vegetable consumption and pancreatic cancer risk in the European Prospective Investigation into Cancer and Nutrition. Int J Cancer *124*, 1926-1934.
- Wachsmann, M. B., Pop, L. M., and Vitetta, E. S. (2012). Pancreatic ductal adenocarcinoma: a review of immunologic aspects. J Investig Med *60*, 643-663.
- Wahi, M. M., Shah, N., Schrock, C. E., Rosemurgy, A. S., 2nd, and Goldin, S. B. (2009). Reproductive factors and risk of pancreatic cancer in women: a review of the literature. Ann Epidemiol *19*, 103-111.
- Walter, K., Omura, N., Hong, S. M., Griffith, M., Vincent, A., Borges, M., and Goggins, M. (2010). Overexpression of smoothened activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. Clin Cancer Res *16*, 1781-1789.
- Wang, Z., Ahmad, A., Li, Y., Azmi, A. S., Miele, L., and Sarkar, F. H. (2011
-). Targeting notch to eradicate pancreatic cancer stem cells for cancer therapy. Anticancer Res *31*, 1105-1113.
- Wei-Guo, H., Xi-Wang, L., and Qi-Ping, L. (2010). Expression of sonic hedgehog signaling pathways in a rat model of chronic pancreatitis. Saudi Med J *31*, 14-17.
- Weterman, M. A., Stoopen, G. M., van Muijen, G. N., Kuznicki, J., Ruiter, D. J., and Bloemers, H. P. (1992). Expression of calcyclin in human melanoma cell lines correlates with metastatic behavior in nude mice. Cancer Res *52*, 1291-1296.
- Whitcomb, D. C. (2004). Inflammation and Cancer V. Chronic pancreatitis and pancreatic cancer. Am J Physiol Gastrointest Liver Physiol *287*, G315-319.
- Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich C, Martin S, Gates LK Jr, Amann ST, Toskes PP, *et al.* (1996). Hereditary

pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet *14*, 141-145.

- Whiteside, T. L. (2008). The tumor microenvironment and its role in promoting tumor growth. Oncogene *27*, 5904-5912.
- Wilentz, R. E., Iacobuzio-Donahue, C. A., Argani, P., McCarthy, D. M., Parsons, J. L., Yeo, C. J., Kern, S. E., and Hruban, R. H. (2000). Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res *60*, 2002-2006.
- Witz, I. P. (2008). Yin-yang activities and vicious cycles in the tumor microenvironment. Cancer Res *68*, 9-13.
- Witz, I. P., and Levy-Nissenbaum, O. (2006). The tumor microenvironment in the post-PAGET era. Cancer Lett *242*, 1-10.
- Wojtowicz-Praga, S. (2003). Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. Invest New Drugs *21*, 21-32.
- Wray, C. J., Ahmad, S. A., Matthews, J. B., and Lowy, A. M. (2005). Surgery for pancreatic cancer: recent controversies and current practice. Gastroenterology *128*, 1626-1641.
- Wu, Y., Li, Y. Y., Matsushima, K., Baba, T., and Mukaida, N. (2008). CCL3-CCR5 axis regulates intratumoral accumulation of leukocytes and fibroblasts and promotes angiogenesis in murine lung metastasis process. J Immunol *181*, 6384-6393.
- Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley, E. R., Graf, T., Pollard, J. W., Segall, J., and Condeelis, J. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res *64*, 7022-7029.
- Xiong, H. Q. (2004). Molecular targeting therapy for pancreatic cancer. Cancer Chemother Pharmacol *54 Suppl 1*, S69-77.
- Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., Kamiyama, M., Hruban, R. H., Eshleman, J. R., Nowak, M. A., *et al.* (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature *467*, 1114-1117.
- Yamamoto, H., Itoh, F., Iku, S., Adachi, Y., Fukushima, H., Sasaki, S., Mukaiya, M., Hirata, K., and Imai, K. (2001). Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human pancreatic adenocarcinomas: clinicopathologic and prognostic significance of matrilysin expression. J Clin Oncol *19*, 1118-1127.
- Yamanaka, Y., Friess, H., Buchler, M., Beger, H. G., Gold, L. I., and Korc, M. (1993). Synthesis and expression of transforming growth factor beta-1, beta-2, and beta-3 in the endocrine and exocrine pancreas. Diabetes *42*, 746-756.
- Yan, H. H., Pickup, M., Pang, Y., Gorska, A. E., Li, Z., Chytil, A., Geng, Y., Gray, J. W., Moses, H. L., and Yang, L. (2010). Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. Cancer Res *70*, 6139-6149.
- Yang J, Yignian Y, Hamrick HE, and Duerksen-Hughes PJ (2003). ATM, ATR, and DNA-PK: Initiators of the cellular genotoxic stress response. Carcinogenesis, (in press).

- Yang, Y. Q., Zhang, L. J., Dong, H., Jiang, C. L., Zhu, Z. G., Wu, J. X., Wu, Y. L., Han, J. S., Xiao, H. S., Gao, H. J., and Zhang, Q. H. (2007). Upregulated expression of S100A6 in human gastric cancer. J Dig Dis *8*, 186-193.
- Yates, C. (2011). Prostate tumor cell plasticity: a consequence of the microenvironment. Adv Exp Med Biol *720*, 81-90.
- Yauch, R. L., Gould, S. E., Scales, S. J., Tang, T., Tian, H., Ahn, C. P., Marshall, D., Fu, L., Januario, T., Kallop, D., *et al.* (2008). A paracrine requirement for hedgehog signalling in cancer. Nature *455*, 406-410.
- Yunis, A. A., Arimura, G. K., and Russin, D. J. (1977). Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. Int J Cancer *19*, 128-135.
- Zapatka, M., Zboralski, D., Radacz, Y., Bockmann, M., Arnold, C., Schoneck, A., Hoppe, S., Tannapfel, A., Schmiegel, W., Simon-Assmann, P., and Schwarte-Waldhoff, I. (2007). Basement membrane component laminin-5 is a target of the tumor suppressor Smad4. Oncogene *26*, 1417-1427.
- Zavoral, M., Minarikova, P., Zavada, F., Salek, C., and Minarik, M. (2011). Molecular biology of pancreatic cancer. World J Gastroenterol *17*, 2897-2908.
- Zimmer DB, Cornwall EH, Landar A, and Song W (1995). The S100 protein family: history, function and expression. Brain Res Bull *37*, 417-429.
- Zucchini, C., Biolchi, A., Strippoli, P., Solmi, R., Rosati, G., Del Governatore, M., Milano, E., Ugolini, G., Salfi, N., Farina, A., *et al.* (2001). Expression profile of epidermal differentiation complex genes in normal and anal cancer cells. Int J Oncol *19*, 1133-1141.
- Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R., and Sorg, C. (1988). Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. Clin Exp Immunol *72*, 510-515.

APPENDIX I: PUBLICATIONS

ARTICLES

LOSS OF SMAD4 EXPRESSION IS ASSOCIATED WITH FEWER S100A8-POSITIVE STROMAL MONOCYTES IN COLORECTAL CANCER AND ATTENUATED CELLULAR RESPONSE TO S100A8 BUT NOT S100A9 IN VITRO
Ang CW, Nedjadi T*, ***Sheikh AA****, Tweedle EM*, Tonack S, Honap S, Jenkins RE, Park BK, Schwarte-Waldhoff I, Khattak I, Azadeh B, Dodson A, Kalirai H, Neoptolemos JP, Rooney PS, Costello E. *Joint authorship
CARCINOGENESIS 2010 Sep; 31(9):1541-51

THE EXPRESSION OF S100A8 IN PANCREATIC CANCER ASSOCIATED MONOCYTES IS ASSOCIATED WITH THE SMAD4 STATUS OF PANCREATIC CANCER CELLS.
Sheikh AA*, Vimalachandran D*, Thompson CC, Jenkins RE, Nedjadi T, Shekouh A, Campbell F, Dodson A, Prime W, Crnogorac-Jurcevic T, Lemoine NR, & Costello E.
PROTEOMICS, June 2007; 7(11):1929-40. *Joint authorship

ABSTRACTS

S100A8 AND S100A9 ACTIVATE CELLULAR SMAD SIGNALLING PATHWAY VIA RAGE IN PANCREATIC CANCER CELLS
Ang CW, ***Sheikh AA***, Tweedle E, Nedjadi T, Tonack S, Honap S, Jenkins R, Park K, Schwarte-Waldhoff I, Dodson A, Kalirai H ,Neoptolemos JP, Rooney PS, Costello E
Pancreatology 2010;10 : 340

CHARACTERISATION OF THE MECHANISMS UNDERLYING THE EFFECTS OF S100A8/S100A9 IN PANCREATIC CANCER
Nedjadi T, Honap S, Ang CW, ***Sheikh AA***, Neoptolemos JP, Costello E
Pancreatology 2010;10 : 340

THE ROLE OF EXOGENOUS S100A8 AND S100A9 PROTEINS AND THEIR RELATIONS TO TUMOURAL SMAD4 STATUS IN COLORECTAL CARCINOGENESIS
Ang CW, ***Sheikh AA***, Tweedle E, Tonack S, Schwarte-Waldhoff I, Neoptolemos J, Rooney P, Costello E.
COLORECTAL DISEASE 2009; 11 (S2): 41.

THE CORRELATION OF TGF-BETA EXPRESSION IN PANCREATIC CANCER AND S100A8/S100A9 EXPRESSION OF STROMAL MONOCYTES
Sheikh AA, Ang C, Tonack S, Nedjadi T, Dodson A, Campbell F, Neoptolemos J, Costello E.
PANCREATOLOGY 2009;9:489

CROSS-TALK BETWEEN CANCER CELLS AND THEIR SURROUNDING HOST CELLS: THE ROLE OF EXOGENOUS S100A8 AND S100A9 AND THEIR RELATIONS TO TUMOURAL SMAD4 STATUS IN CARCINOGENESIS
Ang CW, ***Sheikh AA***, Tweedle EM, Tonack S, Schwarte-Waldhoff I, Rooney P, Neoptolemos J, Costello E.
PANCREATOLOGY 2009;9:445

CHARACTERISATION OF CYTOKINE-MEDIATED CROSSTALK BETWEEN PANCREATIC CANCER AND MONOCYTIC CELLS.
Nedjadi T, ***Sheikh AA***, Ang CW, Neoptolemos JP, Costello E
PANCREATOLOGY 2009;9:487

S100A8 AND S100A9 INCREASE PANCREATIC AND COLORECTAL CANCER CELL MOTILITY AND PROLIFERATION
Sheikh AA, Ang C, Tonack S, Tweedle E, Schwarte-Waldhoff I, Neoptolemos JP, Costello E
PANCREAS, November 2008; 37(4): 496.

PROTEOMIC ANALYSIS OF SERUM AND PANCREATIC JUICE FROM PANCREATIC DUCTAL ADENOCARCINOMA PATIENTS USING ITRAQ LABELLING
Tonack S, Aspinall-O'Dea M, ***Sheikh A***, Jenkins R, Neoptolemos J, Costello, E
PANCREAS, November 2008; 37(4): 498.

LOSS OF SMAD4 EXPRESSION IS RELATED TO THE COMPOSITION OF TUMOUR-ASSOCIATED STROMA AND THE POOR PROGNOSIS IN THE NODE NEGATIVE COLORECTAL CANCER
Tweedle EM, ***Sheikh AA***, Dodson A, Kalirai H, Azadeh B, Rooney P, Costello E
COLORECTAL DISEASE 2008; 10 (S1): 19.

ANALYSIS OF $S100A8^+$, $S100A9^+$ AND $CD68^+$ TUMOUR-ASSOCIATED INFLAMMATORY CELLS IN PANCREATIC CANCER
Sheikh AA, Tonack S, Ang C, Nedjadi T, Dodson A, Campbell F, Neoptolemos JP, Costello E
BRITISH JOURNAL OF SURGERY 2008; 95 (SUPPL 7): 23

THE APPLICATION OF LC-MS AND ITRAQ ON SERUM AND PANCREATIC JUICE SAMPLES FOR PANCREATIC CANCER BIOMARKER DISCOVERY
Tonack S, Aspinall-O'Dea M, ***Sheikh AA***, Jenkins R, Neoptolemos J, Costello E
PANCREATOLOGY 2008; 8(3): 320

INTERACTION BETWEEN PANCREATIC CANCER CELLS AND TUMOUR STROMAL MONOCYTES
Sheikh AA, Vimalachandran D, Campbell F, Dodson A, Neoptolemos JP, Costello E
PANCREAS, November 2007; 35(4): 427.

EFFECTS OF PANCREATIC CANCER ON TUMOUR STROMAL MONOCYTES
Sheikh AA, Vimalachandran D, Campbell F, Dodson A, Neoptolemos JP, Costello E
PANCREATOLOGY, 2007; 7: 244.

THE EXPRESSION OF S100A8 IN TUMOUR-ASSOCIATED MONOCYTES IS DIRECTLY ASSOCIATED WITH THE SMAD4 STATUS OF PANCREATIC CANCER CELLS.
Sheikh AA, Vimalachandran D, Tweedle E, Jenkins RE, Nedjadi T, Shekouh A, Campbell F, Dodson A, Prime W, Lemoine NR, Rooney P, Costello E
PANCREAS, November 2006; 33(4):506,

APPENDIX II: PRESENTATIONS

S100A8 AND S100A9 ACTIVATE CELLULAR SMAD SIGNALLING PATHWAY VIA RAGE IN PANCREATIC CANCER CELLS [P]
Ang CW, ***Sheikh AA***, Tweedle E, Nedjadi T, Tonack S, Honap S, Jenkins R, Park K, Schwarte-Waldhoff I, Dodson A, Kalirai H ,Neoptolemos JP, Rooney PS, Costello E
42nd European Pancreatic Club Meeting, Stockholm, Sweden 16-19 June 2010.

CHARACTERISATION OF THE MECHANISMS UNDERLYING THE EFFECTS OF S100A8/S100A9 IN PANCREATIC CANCER [P]
Nedjadi T, Honap S, Ang CW, ***Sheikh AA***, Neoptolemos JP, Costello E
42nd European Pancreatic Club Meeting, Stockholm, Sweden 16-19 June 2010.
National Cancer Research Institution (NCRI) 2010 annual meeting, Liverpool, UK 7th -10th November 2010

THE ROLE OF EXOGENOUS S100A8 AND S100A9 PROTEINS AND THEIR RELATIONS TO TUMOURAL SMAD4 STATUS IN COLORECTAL CARCINOGENESIS [O]
Ang CW, ***Sheikh AA***, Tweedle E, Tonack S, Schwarte-Waldhoff I, Neoptolemos J, Rooney P, Costello
The European Society of Coloproctology, 23rd-26th September 2009, Prague.

CHARACTERISATION OF CYTOKINE-MEDIATED CROSSTALK BETWEEN PANCREATIC CANCER AND MONOCYTIC CELLS [P]
Nedjadi T, Ang CW, ***Sheikh AA***, Neoptolemos JP, Costello E
41st Meeting of the European Pancreatic Club, Szeged Hungary 1-4 July 2009.
National Cancer Research Institution (NCRI) 2009 annual meeting, Birmingham, UK 7th November to 10th November 2009

THE CORRELATION OF TGF-BETA EXPRESSION IN PANCREATIC CANCER AND S100A8/S100A9 EXPRESSION OF STROMAL MONOCYTES [P]
Sheikh AA, Ang CW, Tonack S, Nedjadi T, Campbell F, Neoptolemos J, Costello E.
41st Meeting of the European Pancreatic Club, Szeged Hungary 1-4 July 2009.

CROSS-TALK BETWEEN CANCER CELLS AND THEIR SURROUNDING HOST CELLS THE ROLE OF EXOGENOUS S100A8 AND S100A9 AND THEIR RELATIONS TO TUMOURAL SMAD4 STATUS IN CARCINOGENESIS [O]
Ang CW, ***Sheikh AA***, Tweedle EM, Tonack S, Schwarte-Waldhoff I, Rooney P, Neoptolemos J, Costello E.
41st Meeting of the European Pancreatic Club, Szeged Hungary 1-4 July 2009.
Northwest & Liverpool Society of Surgeons annual meeting, 5th December 2008

S100A8 AND S100A9 INCREASE PANCREATIC AND COLORECTAL CANCER CELL MOTILITY AND PROLIFERATION [O]
Sheikh AA, Ang C, Tonack S, Schwarte-Waldhoff I, Neoptolemos J, Costello E
American Pancreatic Association 39th meeting, Chicago USA 5th-8th November 2008.

S100A8 INCREASES PANCREATIC AND COLORECTAL CANCER CELL MOTILITY AND PROLIFERATION [P]
Ang CW, ***Sheikh AA***, Tonack S, Rooney P, Neoptolemos JP and Costello E
National Cancer Research Institution (NCRI) 2008 annual meeting, Birmingham, UK 5th-8th October 2008.

PROTEOMIC ANALYSIS OF SERUM AND PANCREATIC JUICE FROM PANCREATIC DUCTAL ADENOCARCINOMA PATIENTS USING ITRAQ LABELLING [P]
Tonack S, Aspinall-O'Dea M, ***Sheikh AA***, Jenkins R, Neoptolemos J, Costello E
American Pancreatic Association 39th meeting, Chicago USA 5-8th November 2008.

CROSS-TALK BETWEEN PANCREATIC CANCER CELLS AND TUMOUR STROMAL MONOCYTES [O]
Sheikh AA, Neoptolemos JP, Costello E.
The College Board, Royal College of Surgeons of England 16th October 2008

ANALYSIS OF S100A8$^+$, S100A9$^+$ AND CD68$^+$ TUMOUR-ASSOCIATED INFLAMMATORY CELLS IN PANCREATIC CANCER [O]
Sheikh AA, Tonack S, Ang CW, Dodson A, Campbell F, Neoptolemos JP, & Costello E
European Pancreatic Club and International Association of Pancreatology, Joint meeting, Lodz Poland, 25th-28th June 2008.
Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland annual meeting, Liverpool UK 25th -26th September 2008.

THE APPLICATION OF LC-MS AND ITRAQ ON SERUM AND PANCREATIC JUICE SAMPLES FOR PANCREATIC CANCER BIOMARKER DISCOVERY [P]
Tonack S, Aspinall-O'Dea M, ***Sheikh AA***, Jenkins R, Neoptolemos J, Costello E
European Pancreatic Club and International Association of Pancreatology, Joint meeting, Lodz Poland, 25th to 28th June 2008.
National Cancer Research Institution (NCRI) 2008 annual meeting, Birmingham, UK 5th October to 8th October 2008.

LOSS OF SMAD4 EXPRESSION IS RELATED TO THE COMPOSITION OF TUMOUR-ASSOCIATED STROMA AND THE POOR PROGNOSIS IN THE NODE NEGATIVE COLORECTAL CANCER [P]
Tweedle E, ***Sheikh AA***, Dodson A, Kalirai H, Azadeh B, Rooney P, Costello E.
American Society of Colorectal Surgery Tripartite meeting Boston USA 7-11th June 2008.
Association of Coloproctology of Great Britain and Ireland 2008 Annual Meeting, Birmingham UK 30th June to 3rd July 2008.

INTERACTION BETWEEN PANCREATIC CANCER CELLS AND TUMOUR STROMAL MONOCYTES [P]
Sheikh AA, Vimalachandran D, Campbell F, Dodson A, Neoptolemos JP, Costello E.
American Pancreatic Association 38th meeting, Chicago USA 1st November 2007

EFFECTS OF PANCREATIC CANCER ON TUMOUR STROMAL MONOCYTES [P]
Sheikh AA, Vimalachandran D, Campbell F, Dodson A, Neoptolemos JP, Costello E.
European Pancreatic Club, Annual Meeting, Newcastle UK 6th-7th July 2007.
National Cancer Research Institution (NCRI) 2007 annual meeting,
Birmingham, UK 30th September to 3rd October 2007.

THE EXPRESSION OF S100A8 IN TUMOUR-ASSOCIATED MONOCYTES IS DEPENDENT ON THE SMAD4 STATUS OF PANCREATIC CANCER CELLS [P]
Sheikh AA, Vimalachandran D, Tweedle E, Shekouh A, Campbell F, Dodson A, Prime W, Crnogorac-Jurcevic T, Lemoine NR, Costello E.
American Pancreatic Association 37th meeting, Chicago USA 5th Nov 2006
National Cancer Research Institution (NCRI) 2006 annual meeting, Birmingham, UK 8th to 11th October 2006
Northwest & Liverpool Society of Surgeons annual meeting, 1st December 2006

APPENDIX III: ORIGINAL PAPERS

RESEARCH ARTICLE

The expression of S100A8 in pancreatic cancer-associated monocytes is associated with the Smad4 status of pancreatic cancer cells

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The cross-talk between tumour cells and the surrounding supporting host cells (stroma) is a key regulator of cancer growth and progression. By undertaking 2-DE analysis of laser capture microdissected malignant and stromal components of pancreatic tumours and benign ductal elements, we have identified high levels of S100A8 and S100A9 in tumour-associated stroma but not in benign or malignant epithelia. Immunohistochemical analysis ($n = 71$ patients) revealed strong expression of both proteins in stromal myeloid cells, subsequently identified as $CD14^+$/$CD68^-$ monocytes/macrophages. Co-immunofluorescence revealed that S100A8 was expressed in a subset of S100A9-positive cells. Correlation of the expression of S100A8 and S100A9 to patient parameters revealed that the microenvironments of tumours which lacked expression of the tumour suppressor protein, Smad4, had significantly reduced numbers of S100A8-immunoreactive ($p = 0.023$) but not S100A9-immunoreactive ($p = 0.21$) cells. The ratio of S100A8- to S100A9-positive cells within individual tumours was significantly lower in Smad4-negative tumours than in Smad4-positive tumours ($p<0.003$). Pancreatitic specimens also contained S100A8- and S100A9-expressing cells, although this was not observed in regions displaying extensive fibrosis. In conclusion, our study provides an extensive analysis of S100A8 and S100A9 in pancreatic disease and highlights a potentially important relationship between pancreatic cancer cells and their surrounding microenvironment.

Received: December 1, 2006
Revised: January 25, 2007
Accepted: February 24 2007

Keywords:
Monocytes / Pancreatic cancer / S100A8 / S100A9 / Smad4

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Abbreviations: IQR, inter-quartile range; **TGF-β**, transforming growth factor-beta; **TMA**, tissue microarrays

1 Introduction

Pancreatic cancer is one of the most aggressive human cancers. Globally it is one of the leading causes of cancer-related deaths, accounting for over 200 000 deaths in the year 2000 alone [1]. A characteristic genetic signature has been eluci-

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dated for pancreatic cancer and includes genetic alterations in K-*ras*, p53, $p16^{INK4a}$ and Smad4 [2]. Surgical resection is still the only realistic form of curative intervention; however, only up to 9% of patients undergo a potentially curative procedure [3], as the disease is often at an advanced stage at the time of diagnosis. Given the currently dismal outlook for patients with pancreatic cancer, considerable effort is being directed towards further identifying cytogenetic characteristics and molecular mechanisms contributing to the disease.

The distinct molecular 'cross-talk' between cancer cells and surrounding host cells is currently being examined in a number of settings [4]. Such signalling can result in modification of the microenvironment by the tumour cells themselves, in order to facilitate tumour invasion and progression. Host stromal cells may also influence tumour behaviour [5, 6] and stimulate tumour proliferation [7]. In addition to influencing tumour behaviour, specific stromal components have also emerged as markers of poor survival in cancer patients [8, 9].

Pancreatic cancer shows a characteristically intense desmoplastic stroma, which can account for up to 75% of the pancreatic tumour volume [10]. It is composed of a number of different host cell types, including fibroblasts, small endothelial-lined vessels and a variety of inflammatory cells, which are both locally derived and recruited from the circulation. Iacobuzio-Donahue *et al.* [11, 12] subdivided the pancreatic host stromal compartments into two distinct regions: juxtatumoural stroma and panstroma. The term 'juxtatumoural stroma' refers to stromal cells that are in direct contact with the tumour, while 'panstroma' refers to all stromal cells within the desmoplastic response to an infiltrating tumour. The pattern of gene expression in these stromal subcompartments was found to be distinct [12], potentially reflecting specific roles for each subcompartment. Whilst pancreatic cancer cells have been demonstrated to both modify [13] and be influenced by their surrounding microenvironment [5, 6], much remains to be elucidated about the interactions between this cancer type and its microenvironment.

We have previously employed proteomic-based studies to determine differential protein expression in pancreatic cancer [14]. In the present study, we employed laser capture microdissection and 2-DE to analyse protein expression in the stromal cells surrounding malignant pancreatic ductal cells. Two of the proteins we found to be preferentially expressed in host stromal cells compared to malignant or benign ductal cells are members of the S100 family of proteins: S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14).

These proteins have been implicated in a variety of inflammatory pathologies, where they have been specifically linked to monocytes and immature macrophages [15, 16]. They can exist as homodimers, although they form stable heterodimeric complexes [17] that are secreted at sites of inflammation [18]. They are thought to have a pro-inflammatory effect, involved in recruitment and migration of inflammatory cells [16, 19, 20].

The S100 family of proteins appears to play a significant role in many cancers. We [14, 21–23] and several other groups [24–27] have previously reported the dysregulated expression of members of the S100 gene family of calcium-binding proteins (*e.g.* S100A6, S100P, S100A4, S100A2 and S100A11) in pancreatic adenocarcinoma. In the current study, we characterised the expression of S100A8 and S100A9 in the pancreatic cancer microenvironment and in so doing, observed a significant association between the number of S100A8 positive cells and the Smad4 status of tumours.

The study was performed with ethical approval from Cheshire and Merseyside Health Authority (Hamilton House, 24 Pall Mall, Liverpool, L3 6AL, UK), LREC ref: 03/02/316A.

2 Materials and methods

2.1 Tissue preparation and laser capture microdissection

For the purposes of laser capture microdissection, frozen tissue was obtained and prepared as described previously [14]. Briefly, pancreatectomy specimens were obtained following surgery, with full ethical consent, examined by a specialist pathologist, cryofixed in liquid isopentane (–160°C) cooled by liquid nitrogen, and stored at –80°C. A histological diagnosis of pancreatic ductal adenocarcinoma was necessary for inclusion in the study. For staining, 7-μm-thick frozen sections were cut onto slides (that were pre-cleaned using detergent, washed with deionised water and oven-dried at 40°C) using a Bright OTF 5000 cryostat (chamber temperature –25°C). Sections were placed on dry ice or kept in the cryostat chamber prior to staining. Haematoxylin and eosin staining [14] was carried out only for monitoring of tissue sections. Methyl green staining of the sections used for laser capture microdissection was as follows: sections were fixed (using 70% ethanol for 1 min), washed in deionised water for 15–30 s, stained with violet-free methyl green (2% w/v in deionised water) for 30 s, rinsed twice in deionised water and dehydrated (70% ethanol for 30 s, 95% ethanol for 30 s, 2 × 100% ethanol for 30 s, xylene 2 × for 5 min). Complete protease inhibitor cocktail tablets (Roche) were added to the staining solutions (one tablet/80 mL solution). Following staining with methyl green, sections were air-dried and microdissected using an Arcturus PixCell II system (Arcturus, Mountain View, CA, USA) with a 7.5-μm laser beam. Power and pulse duration were typically set at 70 mW and 3–10 ms, respectively. An estimated 50 000 cells were laser captured using an average of 25 000 pulses (with the exception of proteins separated in Figs. 1E and F, which were derived from an estimated 150 000 laser captured cells). In each case of stroma examined ($n = 3$), the stromal compartment was subdivided into two separate regions for microdissection, stromal cells adjacent to cancer cells denot-

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ed as juxtatumoural stroma, and all other stroma denoted panstroma. A total of four dissected benign and four dissected malignant samples were analysed. In two cases, both the benign and malignant samples were form the same patient, and in one case the malignant and stromal sample were derived from the same patient.

2.2 Protein extraction and 2-D separation

Proteins were extracted from microdissected cells in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 1% w/v DTT). Accurate protein quantification using conventional protein assays was rendered difficult due to the minute quantities of protein extracted from 50 000 laser capture cells as well as the incompatibility of the lysis buffer with such assays. However, normalisation for the purposes of even gel loading was necessary and was undertaken as described in detail previously [14]. Briefly, sample protein concentrations were estimated relative to a reference sample lysate. Twofold dilution series, from undiluted to a 1-in-16 dilution of the test samples and reference sample, were prepared in lysis buffer, mixed with two volumes of sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 25% w/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol) containing a trace of bromophenol blue, heated at 95°C for 10 min and both test and reference samples subjected to 1-D SDS-PAGE on small format gels (10% separating and 4% stacking gels) at a constant voltage of 150 V. Gels were silver-stained, densitometrically scanned and analysed using TotalLab software (NonLinear Dynamics) to inform our estimation of relative protein amounts in the test samples compared to the reference sample.

2.3 Gel alignment for spot picking and protein identification

The separation of proteins from an estimated 50 000 laser capture-procured cells yielded insufficient protein for identification by MS. Therefore, for the identification of S100A8 and S100A9, alignment of gels was undertaken stepwise as follows: Silver-stained gels displaying proteins from 50 000 laser capture-procured stromal cells were carefully aligned with silver-stained gels containing protein from an estimated 150 000 laser capture-procured tumour cells. The latter were subsequently aligned with silver-stained gels containing protein from one complete undissected stroma-rich tumour tissue section and this gel was aligned with a CBB -stained gel containing protein from 30 complete, stroma-rich tumour tissue sections. In this way landmark reference protein spots were established, facilitating the location of protein spots of interest. Following spot picking, in-gel trypsin digestion was then performed and in the case of S100A9, a peptide mass fingerprint was obtained on a MALDI-TOF mass spectrometer (Voyager DE Pro, Applied Biosystems, Framingham, USA) and resultant mass lists searched against the NCBI database using MASCOT (Matrix Science), with a peptide tolerance of 100 ppm. In the case of S100A8, LC-MS/MS analysis was necessary. The tryptic digest was delivered into a QSTAR® Pulsar i hybrid mass spectrometer (Applied Biosystems) by automated in-line RP-LC (integrated LCPackings System, 5 mm C18 μ-precolumn cartridge and 75 μm × 15 cm C18 column, Dionex, Camberley, UK) *via* a nano-electrospray source head and a 10-μm id PicoTip (New Objective, Woburn, USA). A gradient from 5% ACN/0.05% TFA v/v to 48% ACN/0.05% TFA v/v in 60 min was applied at a demanded flow rate of 200 nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (Analyst® software, Applied Biosystems). Data were again submitted to MASCOT and the NCBI database was searched with the MS tolerance set to 1.2 Da and the MS/MS tolerance to 0.6 Da, with no modifications allowed.

2.4 Immunohistochemical staining

Immunohistochemical staining of three tissue microarrays (TMAs) was undertaken essentially as described previously [14]. The TMAs were as follows: (i) a pancreatic cancer (pancreatic ductal adenocarcinoma) microarray containing duplicate malignant specimens from 71 patients along with matched benign tissue from 53 patients (248 cores in total), and eight cores each of normal colonic, liver and kidney samples; (ii) a chronic pancreatitis tissue array containing cores representing 24 chronic pancreatitis specimens plus 24 normal pancreas specimens, each arrayed in duplicate (96 cores in total) with five cores each of normal colonic, liver and kidney samples.

Five-micron-thick sections were deparaffinised in xylene and then rehydrated through alcohol to distilled water. Antigen was retrieved by pressure-cooking the slides in 10 mM EDTA (pH 7.0) for 3 min. Immunohistochemical staining was performed using an automatic staining system (Universal Staining System, DAKO). Slides were incubated for 1 h with polyclonal goat anti-human S100A8 (0.1 μg/mL; Santa Cruz Biotechnology) or polyclonal rabbit anti-human S100A9 (0.2 μg/mL; Santa Cruz Biotechnology), or an mAb raised against human Smad4 (clone B8; 2.0 μg/mL, Santa Cruz Biotechnology), rinsed in PBS and the antibody localisation visualised by incubating sections with a HRP-conjugated secondary antibody for 30 min followed by diaminobenzidine (DAKO) for 10 min. Slides were counterstained with haematoxylin for 2 min, dehydrated with 100% ethanol and xylene and coverslips mounted with D.P.X. mountant (BDH). Negative controls were incubated with the labelled secondary antibodies only. Both TMAs were scored in a similar manner by specialist histopathologists. For S100A8 and S100A9, the numbers of positive cells *per* core were counted at a magnification of 40 ×. For Smad4, negative cases were defined as the complete absence of expression, as described previously [28].

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2.5 Immunofluorescence

Sections were de-waxed in xylene and rehydrated in graded ethanol to distilled water. Heat-mediated antigen retrieval was performed in a pressure cooker containing 10 mM/L EDTA (pH 7.4) in which sections were treated at full pressure for 3 min. Manual staining was then undertaken in a flat-bed incubation tray. Primary antibodies (polyclonal goat anti-human S100A8 (0.1 μg/mL; Santa Cruz Biotechnology) or polyclonal rabbit anti-human S100A9 (0.2 μg/mL; Santa Cruz Biotechnology) or anti-CD14 (monocyte/macrophage marker; diluted 1:200), or anti-CD68 (mature macrophage marker; diluted 1:4000) or anti-smooth muscle actin (smooth muscle/myofibroblast marker; diluted 1:50) or anti-desmin (smooth muscle/myofibroblast marker; diluted 1:40) or anti-vimentin (diluted 1:200), or anti-CD20 (B lymphocyte marker; diluted 1;4000) or anti-CD34 (endothelial marker; diluted 1:50) or anti-CD38 (plasma cell marker; diluted 1:100) or anti-CD3 (T-cell marker; diluted 1:100) or anti-CD79A (B-cell marker; diluted 1:500) or anti-mast cell trypsin (mast cell marker; diluted 1:400) were applied singly or concurrently in pairs for 60 min, after which sections were washed in TBS and incubated for 60 min with a mixture of two fluorescently labelled secondary antibodies: *e.g.* FITC-labelled donkey anti-goat IgG (30 μg/mL; Santa Cruz Biotechnology) and TRITC-labelled swine anti-rabbit Ig's (25 μg/mL; DakoCytomation) for detection of S100A8 and S100A9, respectively. Sections were washed in TBS and mounted in aqueous mounting medium containing DAPI (Vector Laboratories, Peterborough, UK). Negative controls were performed using antibody diluent only in place of primary antibody.

2.6 Statistical analyses

All statistical analyses were performed using Statview version 5.01 (SAS Institute). Results were considered significant for p-values <0.05. To evaluate the extent of S100A8 and S100A9 expression in pancreatic tumour-associated stroma, the number of positively stained cells in each TMA core was determined and the mean number of positive cells *per* duplicate patient core calculated. For the purpose of analysis, patients were categorised into two groups: those with S100A8-positive cell counts >median (high S100A8) and those with S100A8-positive cell counts ≤median (low S100A8). Groups were similarly established for high and low S100A9. Continuous variables were compared using the Mann–Whitney U or the Wilcoxon signed rank test. Associations between the number of S100A8- or S100A9-positive inflammatory cells and each of the clinicopathologic parameters below were established by cross-tabulating data and applying Fisher's two-sided exact test, (χ^2-test for tumour grade). Clinical data were available for gender, age at surgery, lymph node status, resection margin status, tumour size (<20mm *versus* ≥20mm), tumour grade, and presence of vascular and peri-neural invasion. To examine a correlation between S100A8- or S100A9-positive inflammatory cells and patient survival time, life tables were constructed from survival data and Kaplan–Meier curves plotted. Comparisons between groups were performed using the log-rank test. Survival time was measured from date of initial surgery to date of death, counting death from any cause as the end point or the last date of information as the end point if no death was documented.

3 Results

3.1 Proteomic analysis of dissected pancreatic compartments revealed differences in the intensities of spots denoted X and Y

Using a 2-DE approach, we previously reported differential protein expression between laser capture-procured populations of benign and malignant pancreatic ductal epithelial cells [14]. Further proteomic analyses comparing similar quantities of microdissected tumour-associated stroma with benign or malignant ductal cells were performed. Specifically, comparisons of similarly loaded silver-stained gels produced with protein from approximately 50 000 laser capture-procured benign ductal epithelial cells ($n = 3$ patients), malignant cells ($n = 3$ patients), juxtatumoural stroma cells ($n = 3$ patients) and panstroma cells ($n = 3$ patients) was undertaken. An example of each gel type is shown in Figs. 1A–D. Analysis of all 12 2-D gels revealed two spots (labelled X and Y, Fig. 1) with higher intensities in gels containing stromal-derived proteins compared to the gels containing proteins from benign or malignant ductal cells. Spot X (denoted by squares in Fig. 1) was detected on both juxtatumoural and panstromal gels derived from all three patients (examples are shown in Figs. 1C and D). The spot intensities between juxtatumoural and panstromal gels for samples derived from the same patients were similar, indicating no detectable difference in the expression of this protein between juxtatumoural stroma and panstroma. In contrast, spot X was either undetectable (Figs. 1A and B) in benign ($n = 2$) and malignant cases ($n = 2$) or scarcely detectable in benign and malignant cases ($n = 1$ sample taken from same patient, not shown in the figure). Similarly, spot Y (denoted by circles in Fig. 1) was detected, with similar intensities on juxtatumoural and panstromal gels (see Figs. 1C and D), which were derived from two patients. Spot Y was undetectable in all benign ($n = 3$) and malignant ($n = 3$) cases examined. In one instance, juxtatumoural stroma, panstroma and malignant cells were derived from the same patient. In this case, both spots X and Y were detected on gels containing stroma-derived protein (juxtatumoural stroma and panstroma), while neither protein spot was detected in the gel containing protein from malignant cells.

The gels produced with protein from approximately 50 000 laser capture-procured cells described above were compared with gels (Figs. 1E and F) produced with protein from approximately 150 000 laser capture-procured benign

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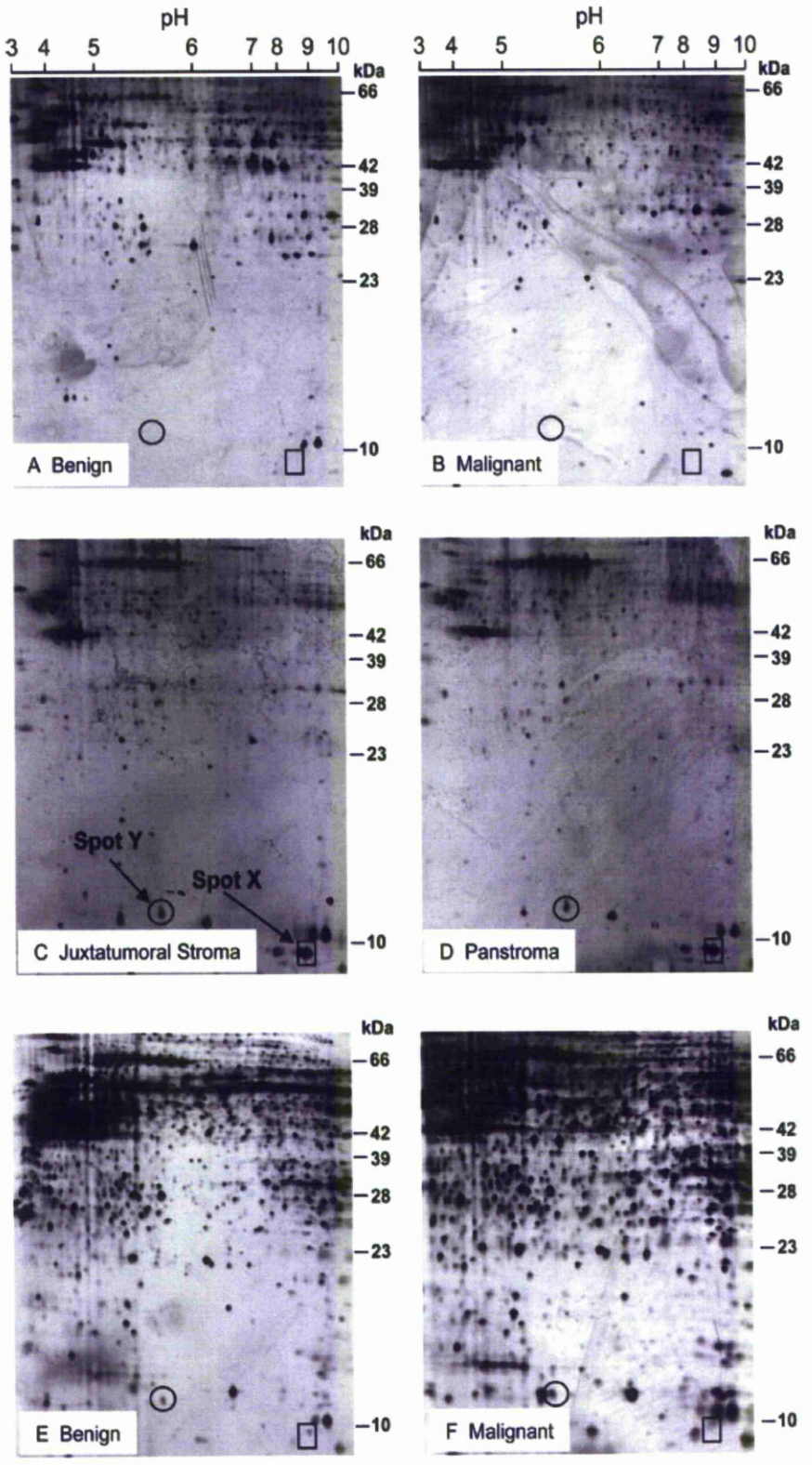

Figure 1. Silver-stained gel images containing proteins extracted from 50 000 laser capture-procured benign (A), malignant (B), juxtatumoural (C) and panstromal (D) cells, as indicated. Panels E and F represent silver-stained gel images containing proteins extracted from approximately 150 000 laser capture-procured benign (E) or malignant cells (F). The juxtatumoural and panstromal cells used in panels C and D were patient-matched, as were the benign and malignant cells in panels E and F.

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and malignant cells which were derived from the same patient. While both the X and Y protein spots were detected on these gels, they were comparatively weakly stained despite the increased protein load, compared to their appearance on stromal gels.

3.2 S100A8 and S100A9 are overexpressed in tumour-associated stroma

Spots X and Y were not definitively identified from silver-stained gels containing protein from approximately 50 000 laser capture-procured cells, due to insufficient material. Therefore, such gels were aligned with bulk-loaded CBB-stained gels and proteins identified from the latter. Alignment of gels was undertaken stepwise, starting with silver-stained gels with low protein loads, and progressing to silver-stained gels containing protein from one undissected stroma-rich tumour tissue section and finally aligning with a CBB-stained gel containing protein from 30 undissected stroma-rich tumour tissue sections. This enabled landmark reference protein spots to be established, facilitating the location of the protein spots of interest, which were subsequently picked up from the bulk-loaded gel. Following LC-MS analysis, three peptides were detected for spot X (GNFHAVYR, ALNSIIDVYHK and carboxamidomethylated LLETECPQYIR), resulting in an overall MOWSE score of 157 and a sequence coverage of 31%. The protein was identified as S100A8 or calgranulin A/MRP 8 (NCBI accession number CAG28602). S100A8 is small (10.9 kDa) and basic (p*I* 9.19) due to an unusually high occurrence of lysine residues (12.9%), so that obtaining a higher sequence coverage using trypsin as the digestion reagent would be difficult. MALDI-MS led to the detection of four peptides for spot Y (DLQNFLK, LGHPDTLNQGEFK, NIETIINTFHQYSVK, MHEGDEGPGHHHKPGLGEGTP), resulting in an overall MOWSE score of 397 and a sequence coverage of 49%. This protein was identified as S100A9 or calgranulin B/MRP 14 (NCBI accession number CAG47020), which is also small (13.2 kDa) and has a p*I* of 5.7. Since the gels from which spots were picked for identification contained both tumour and stromal proteins, the possibility that the proteins identified were not the same proteins as those present in spots X and Y on stromal gels loaded with laser capture-procured material could not formally be ruled out. Thus, validation of our proteomic findings was sought using an independent technique, described in the following sections.

3.3 Immunohistochemical analysis of pancreatic cancer specimens confirmed high S100A8 and S100A9 expression in cells of tumour-associated stroma

Immunohistochemical staining of a pancreatic cancer TMA for S100A8 resulted in no specific immunostaining of either benign or malignant epithelial cells (Figs. 2A–C). By contrast, strong S100A8 immunostaining was readily detected in

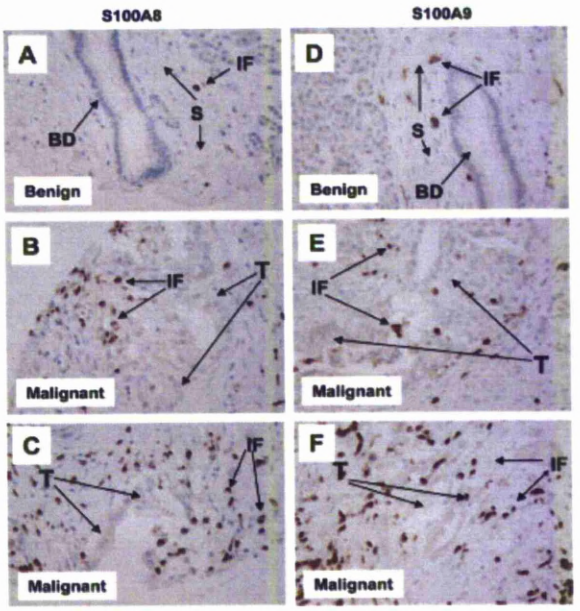

Figure 2. Photomicrographs showing selected sections of benign and malignant tissue, as indicated, from a pancreatic cancer microarray, immunohistochemically stained for S100A8 (left panel) and S100A9 (right panel). BD = benign duct, IF = inflammatory cell, S = stroma, T= tumour cell. Examples of cancer tissues with low (B, E) and high (C, F) numbers of positively stained inflammatory cells are shown.

the inflammatory cells of tumour stroma (Figs. 2B and C). Some tumours contained a limited number of S100A8-positive inflammatory cells (Fig. 2B, see below for a detailed analysis), while others displayed a high density of S100A8-positive cells infiltrating the tumour (Fig. 2C). S100A9 displayed a very similar pattern of expression to that of S100A8 (Figs. 2D–F). It was not detected in benign or malignant epithelial cells; however, distinct S100A9 immunostaining was evident in inflammatory cells in the stromal component of all tumours (71/71).

3.4 S100A8 is detectable in a subset of S100A9-positive cells

Immunofluorescent double staining experiments on pancreatic cancer specimens showed co-localisation of S100A8 or S100A9 with CD14, a monocyte/macrophage marker. Neither of the S100 proteins was co-expressed with CD68, a marker of mature macrophages (not shown). Moreover, cellular markers of smooth muscle, myofibroblasts, lymphocytes, T- and B-cells, endothelial cells, mast cells and plasma cells also failed to co-localise with S100A9 (not shown).

To determine whether S100A8 and S100A9 were co-expressed in the same cells, additional immunofluorescent double staining experiments were performed ($n = 3$ independent pancreatic cancer cases). Serial sections were incu-

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bated separately or concurrently with polyclonal goat anti-S100A8 and/or polyclonal rabbit anti-S100A9. In all cases, this was followed by the application of two fluorescently labelled secondary antibodies (*i.e.* the FITC-labelled donkey anti-goat IgG for the detection of the S100A8 antibody and the TRITC-labelled swine anti-rabbit Ig's for the detection of the anti-S100A9 antibody). This enabled the detection of any non-specific cross-reactivity of secondary antibodies. Under conditions of respective single labelling (Figs. 3A and B), S100A8 immunoreactive cells (green, Fig. 3A) and S100A9 immunoreactive cells (red, Fig. 3B) were clearly visible and were confined to the stroma. Cross-reactivity between primary goat and secondary rabbit or primary rabbit and secondary goat antibodies was not detected (Figs. 3A and B, respectively). Tumour cells were unstained. Dual staining (Fig. 3C) revealed the presence of cells expressing both S100A8 and S100A9 (yellow). Under the conditions of dual staining, a number of cells showed expression of S100A9 only (red). However, cells expressing S100A8 only were not detectable. An example of a tumour in which the majority of cells are co-labelled is also shown (Fig. 3D). Thus, S100A8 was detected in a subset of S100A9-positive cells contained within the stromal compartment of pancreatic sample.

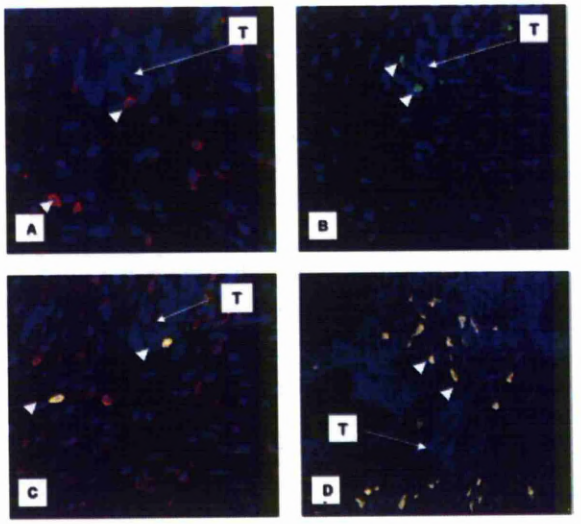

Figure 3. Pancreatic cancer sections were stained for (A) immunofluorescence analysis with anti-S100A9-TRITC (red), (B) anti-S100A8-FITC (green), (C) co-stained with anti-S100A8-FITC and anti-S100A9-TRITC in a patient with a low S100A8-to-S100A9 ratio, where A–C were serial sections from the same patient, (D) co-stained with anti-S100A8-FITC and anti-S100A9-TRITC in a patient with a high S100A8-to-S100A9 ratio.

3.5 Quantification of S100A8 and S100A9 staining and correlation with patient parameters

To evaluate the extent of S100A8 and S100A9 expression in pancreatic tumour-associated stroma, the number of positively stained cells in each tumour core of the immunostained cancer microarray was determined and the mean number of positive cells *per* duplicate patient core plotted (Fig. 4). Tumours contained significantly lower numbers of S100A8-positive cells than S100A9-positive cells, as evidenced by a median number of S100A8-positive cells of 15.0 (inter-quartile range (IQR) 5.2–43.7) compared to a median number of 35 (IQR 20–74.5) of S100A9-positive cells ($p<0.0001$, Wilcoxon signed rank test). Overall, tumours contained 0.28- to 16-fold (median 2.2, IQR 1.48–4.5) greater numbers of S100A9-positive cells than S100A8-positive cells.

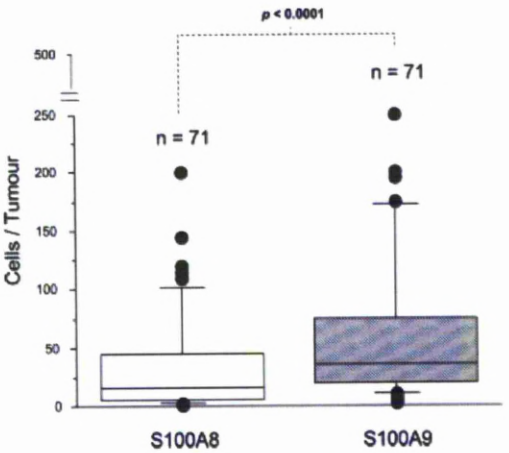

Figure 4. Mean S100A8- and S100A9-positive cell counts in pancreatic cancer specimens from 71 patients shown as box plots. The *p*-value shown is for comparison of the median number of cells positive for S100A8 and S100A9 using the Wilcoxon signed rank test.

For the purposes of examining associations between the number of S100A8- or S100A9-immunopositive cells and other patient parameters, patients were categorised into two groups, *i.e.* those having positive cell numbers less $\leq$ to the median or $>$ than the median for the respective proteins. A strong positive correlation was observed between the number of S100A8- and S100A9-positive cells (Fisher's exact test, $p = 0.0001$). For the parameters of gender, age at surgery, nodal metastases, tumour size and grade, data for 71 patients were available. Resection margin status, vascular invasion and peri-neural invasion data were available for 63, 65 and 68 patients, respectively. No associations were observed between the numbers of stromal associated S100A8- or S100A9-positive cells and these clinicopathologic parameters (Table 1). Survival data for all 71 patients, three of whom were alive at the time of analysis, were available. Kaplan–Meier analysis indicated that the number of S100A8- or S100A9-positive cells did not correlate with patient survival (Log-rank *p* value, 0.72 and 0.49 for S100A8 and S100A9, respectively).

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Table 1. Pancreatic cancer: S100A8- and S100A9-positive cells and association with clinicopathologic parameters

Parameter	All cases $n = 71$ (%)	Low S100A8$^+$ (≤median) $n = 36$ (%)	High S100A8$^+$ (>median) $n = 35$ (%)	Signif.	Low S100A9$^+$ (≤median) $n = 39$ (%)	High S100A9$^+$ (>median) $n = 32$ (%)	Signif.
Gender							
Male	43 (61)	25 (69)	18 (51)	ns[a]	23 (59)	20 (63)	ns[a]
Female	28 (39)	11 (31)	17 (49)		16 (41)	12 (37)	
Age at surgery							
<60 years	20 (28)	7 (19)	13 (37)	ns[a]	7 (18)	13 (40)	ns[a]
>60 years	51 (72)	29 (81)	22 (63)		32 (82)	19 (60)	
Tumour size							
<20 mm	22 (31)	13 (36)	9 (26)	ns[a]	13 (33)	9 (28)	ns[a]
>20 mm	49 (69)	23 (64)	26 (74)		26 (67)	23(72)	
Tumour grade							
Poorly dif.	25 (35)	13 (36)	12 (34)		15 (38)	10 (31)	
Moderate dif.	36 (51)	15 (42)	21 (60)	ns[b]	17 (44)	19 (59)	ns[b]
Well dif.	10 (14)	8 (22)	2 (6)		7 (18)	3 (10)	
Nodal metastases							
Present	57 (80)	28 (78)	29 (83)	ns[a]	37 (79)	26 (81)	ns[a]
Not present	14 (20)	8 (22)	6 (17)		8 (21)	6 (19)	
Involved resection margin ($n = 63$)							
Yes	42 (59)	22 (61)	20 (57)	ns[a]	24 (67)	18 (56)	ns[a]
No	21 (30)	9 (25)	12 (34)		10 (26)	17 (34)	
Not recorded ($n = 8$)	8 (11)	5 (14)	3 (9)		5 (13)	3 (10)	
Vascular invasion ($n = 65$)							
Present	54 (76)	28 (78)	26 (74)		32 (82)	22 (69)	
Not present	11 (16)	5 (14)	6 (17)	ns[a]	4 (10)	7 (22)	ns[a]
Not recorded ($n = 6$)	6 (8)	3 (8)	3 (9)		3 (8)	3 (9)	
Neural invasion ($n = 68$)							
Present	65 (92)	33 (92)	32 (91)		37 (95)	28 (88)	
Not present	3 (4)	1 (3)	2 (6)	ns[a]	0 (0)	3 (9)	ns[a]
Not recorded ($n = 3$)	3 (4)	2 (5)	1 (3)		2 (5)	1 (3)	

All values shown in parentheses are percentages

a) Fisher's two-sided exact test (significance set at $p<0.05$)

b) χ^2 test (significance set at $p<0.05$); ns = not significant

3.6 Loss of Smad4 expression is associated with reduced numbers of S100A8- but not S100A9-positive inflammatory cells

Smad4 expression data were available for 64 patients, of whom 29 patients (45%) were Smad4 negative. The number of S100A9-positive cells was independent of the Smad4 status of tumours (Table 2: $p = 0.21$ Fisher's exact test). By contrast, Smad4-negative tumours were associated with low numbers of S100A8-positive cells ($p = 0.023$, Table 2). When the mean number of S100A8-positive cells *per* tumour was examined relative to Smad4 status (Fig. 5A), Smad4-negative tumours had significantly fewer S100A8-positive cells (median 8, IQR 3.0–20) compared to Smad4-positive tumours (median 20.5, IQR 9–49; $p = 0.01$, Mann-Whitney *U*-test). Such a relationship was not established for S100A9-positive cells and Smad4 status (Fig. 5B). The median number of S100A9-positive cells in Smad4-negative tumours was 28 (IQR 18.7–60), compared to a median of 40 (IQR 23.5–98.2) in Smad4-positive tumours ($p = 0.21$, Mann-Whitney *U*-test). To examine the relationship between S100A8- and S100A9-immunoreactive cells within each individual patient tumour, the ratio of S100A8- to S100A9-positive cells was assessed for each tumour (Table 2). A striking difference between Smad4-positive and Smad4-negative tumours was observed (Fig. 5C). A median S100A8:S100A9 ratio of 0.60 (IQR 0.325–0.825) in Smad4-positive tumours was observed compared with a significantly lower median S100A8:S100A9 ratio of 0.316 (IQR 0.15–0.466) in Smad4-negative tumours ($p<0.003$, Mann-Whitney *U*-test). Thus, a strong positive

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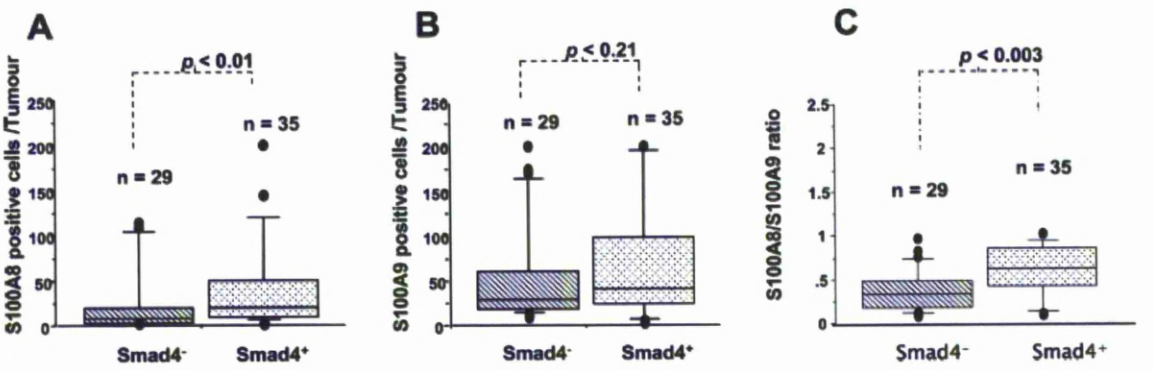

Figure 5. The mean numbers of S100A8- (A) or S100A9-positive cells (B) or the ratio of S100A8:S100A9 (C) *per* patient were plotted for Smad4-negative and Smad4-positive tumours. The *p*-value shown is for comparison (using the Mann-Whitney *U*-test) of the median number of S100A8- or S100A9-positive cells for Smad4-negative and Smad4-positive tumours. Outliers (with >250 S100A9-positive cells) are not shown, but were included in the analyses.

Table 2. Smad4 expression and association with S100A8- and S100A9-positive cells counts

Smad4 expression	All cases	Low $S100A8^+$ cells	High $S100A8^+$ cells	Signif.	Low $S100A9^+$ cells	High $S100A9^+$ cells	Signif.	Median number of cells $S100A8^+/S100A9^+$	Signif.
Pancreatic cancer ($n = 64$)	$n = 71$	$n = 36$	$n = 35$		$n = 39$	$n = 32$			
Positive	35	19	10	**0.023**[a]	18	19	0.21	0.60	**0.003**[b]
Negative	29	12	23		16	11		0.316	
Not recorded	7	5	2		5	2			

a) Fisher's two-sided exact test (significance set at $p<0.05$)
b) Mann Whitney test (significance set at $p<0.05$)

relationship between the loss expression of Smad4 in tumours cells and the lack of expression of S100A8 in stromal inflammatory cells was established.

3.7 S100A8- and S100A9-positive inflammatory cells are detectable in tissue affected by chronic pancreatitis

To examine whether S100A8 and S100A9 are expressed in tissue affected by chronic pancreatitis, a pancreatitis tissue array was immunostained for the detection of both proteins. Normal tissue contained few S100A8- (Fig. 6A) or S100A9-positive cells (Fig. 6E). By contrast, in tissue affected by chronic pancreatitis, inflammatory cells positive for S100A8 (Figs. 6B and C) and S100A9 (Figs. 6F and G) were readily detected. A median S100A8:S100A9 ratio of 0.723 (IQR 0.508–1.08) was observed ($n = 23$ cases). The exception was extensive fibrotic elements of chronic pancreatitis tissue which contained few cells positive for either S100A8 or S100A9 (Figs. 6D and H, respectively). Benign ductal cells were negative for both proteins, as before.

4 Discussion

In this study, we have uncovered a relationship between the phenotype of monocytes/macrophages in the microenvironment of pancreatic cells and the Smad4 status of the corresponding tumour cells. Our study highlights the complex interaction between tumour cells and the surrounding host tissue.

We initially identified S100A8 and S100A9 as predominantly stromal proteins through comparison of laser capture-procured pancreatic stromal cell protein and benign or malignant epithelial cell protein. Two recent proteomics studies, both performed with undissected bulk pancreatic tissue [29, 30], reported high levels of S100A8 and S100A9, respectively, in bulk pancreatic cancer tissue compared to bulk normal pancreas tissue. Our study is consistent with these reports, although it pinpoints the high-level expression of these proteins to the tumour-associated stroma. While proteomic analysis of microdissected material provides valuable information, its labour-intensive nature, combined with the restriction to readily dissectible specimens, limited its use to a small number of cases. Therefore, an independent

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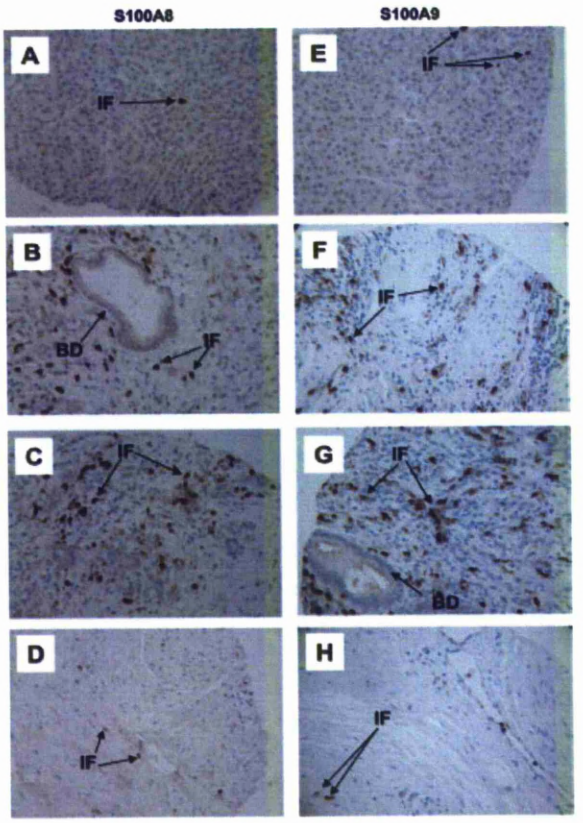

Figure 6. Photomicrographs showing selected sections of normal (A and E) and pancreatitic tissue (B–D, F–H), from a microarray of chronic pancreatitis tissue, immunohistochemically stained for S100A8 (left) and S100A9 (right). BD = benign duct, IF = inflammatory cell. Panels D and H show fibrotic areas labelled with S100A8 and S100A9, respectively.

approach, namely, immunohistochemistry, enabling rapid analysis of larger numbers of samples was subsequently adopted. Our immunohistochemical analysis confirmed the presence of high levels of both S100A8 and S100A9 in specific cells of stroma compared to either benign or malignant epithelial elements. Of note, neither the proteomic nor the immunohistochemical approach indicated a significant difference in the expression of S100A8 or S100A9 between juxtatumoural stroma and panstroma.

The cells strongly positive for S100A8 or S100A9 were identified as monocytes/immature macrophages (*i.e.* $CD14^+/CD68^-$), while strongly positive neutrophils were also observed in blood vessels around the tumour (not shown). This is consistent with the reported abundant expression of these proteins in cells of the myeloid lineage, [15] namely, granulocytes, monocytes, and immature macrophages. Specifically, expression has been documented to be restricted to the early stages of monocyte differentiation and not observed in resting mature tissue macrophages [16, 31, 32].

The precise function of S100A8 and S100A9 in the pancreatic cancer tumour microenvironment is unclear, although their expression and secretion are likely to contribute to the host inflammatory response to the tumour. These proteins have been implicated in a variety of chronic inflammatory conditions such as cystic fibrosis, rheumatoid arthritis, tuberculosis and transplant rejection [15, 16]. They form stable heterodimeric complexes (also known as calprotectin) at sites of inflammation [16, 17] and are highly chemotactic, contributing to host inflammatory responses such as leukocyte trafficking, adhesion and migration [16, 19, 20]. They also promote phagocyte migration by enhancing the polymerisation of microtubules [33, 34], and S100A8/S100A9 heterodimers are believed to recruit further monocytes to sites of inflammation [35]. The results of a recent study [36] suggest that the expression of S100A8/S100A9 was induced in the lungs of mice by the presence of distant primary tumours in the same animals. This in turn attracted myeloid cells to the lungs and ultimately supported the invasion of tumour cells to this site. The study showed that factors such as vascular endothelial growth factor-A as well as transforming growth factor-beta (TGF-β) induced the expression of S100A8 and S100A9. The study supports a role for these proteins in the pre-metastatic phase of cancer dissemination.

While no significant associations were observed between the number of S100A8 or S100A9 positive cells in the pancreatic tumour environment and clinical parameters of disease, a major finding from our TMA analysis was that the number of S100A8-positive inflammatory cells in the stroma was linked to the Smad4 status of tumour cells. Smad4 is an intracellular mediator of TGF-β signalling [37]. Loss of Smad4 expression is observed in around 50% of pancreatic cancers [38] and is a relatively late event in pancreatic cancer development, which presumably allows tumour cells to escape the growth suppressive and pro-apoptotic effects of TGF-β [39, 40]. While the exact consequences of Smad4 loss on pancreatic cancer are unknown, its loss from pancreatic cancer cells appears primarily to affect the interaction between cancer cells and the tumour microenvironment rather than affecting the growth of the cancer cells themselves. Schwarte-Waldhoff *et al.* [41] showed that restoration of Smad4 in pancreatic cancer cells suppressed tumour formation *in vivo*, by inhibiting angiogenesis in the tumour microenvironment. An independent study confirmed the reduction in angiogenesis following re-expression of Smad4, and also reported reduced invasion [42]. A recent study has shown that knock-down of Smad4 rendered cancer cells resistant to TGF-β-induced cell cycle arrest and migration but not to TGF-β-induced epithelial-mesenchymal transition [43], indicating that loss of Smad4 appeared to abolish TGF-β-mediated tumour suppressive functions, while maintaining some TGF-β-mediated tumour-promoting functions [44]. Pancreatic cancer cells have been shown to overexpress TGF-β [37], which, in turn, is likely to affect the tumour microenvironment [13] influencing the composition of the extracellular matrix. Although it is unclear how the Smad4 status

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of the cancer influences S100A8 expression, a murine model of wound healing shows that TGF-β negatively regulates the expression of S100A8 in fibroblasts [45]. Future studies will be required to unravel the relationship between the Smad4 status of tumour cells and the expression of S100A8 in the tumour monocytes. Interestingly, we did not observe a statistically significant link between S100A9-positive cell numbers and Smad4 tumour status. Thus the overall number of monocytes/immature macrophages present in the environment of tumours appeared to be independent of the Smad4 status. The expression of S100A8 and S100A9 is believed to change as monocytes, recruited from the blood stream to sites of inflammation, differentiate to mature macrophages [46]. They initially express both S100A8 and S100A9 and as they mature, S100A8 expression ceases, leaving only S100A9, which is also subsequently lost as the cell matures further [46]. Our co-immunofluorescence data are consistent with the presence of S100A8 in a subset of S100A9-positive pancreatic cancer stromal cells. Evidence has been provided for the presence of S100A8-S100A9 heterodimeric complexes during the early stages of monocyte/macrophage maturation and their expression has been shown to correlate with the intensity of the inflammatory process [32]. It is thus possible that the process of maturation of monocytes to macrophages in the pancreatic cancer tumour microenvironment is influenced by the Smad4 status of tumour cells.

Finally, our study has important implications for the potential use of S100A8 and S100A9 as markers of pancreatic cancer. The overexpression of S100A8 and S100A9 in certain cancer types has focussed much recent interest on their use as tumour markers. S100A9 expression has been reported in immunohistochemical studies of hepatocellular carcinomas, pulmonary adenocarcinomas and invasive ductal carcinomas of the breast [47–49]. Expression of both S100A8 and S100A9 has been observed by immunohistochemical studies in invasive squamous cell carcinoma of the uterine cervix and in prostate cancer [50, 51]. In the case of the latter [51], S100A9 levels were significantly elevated in serum from prostate cancer patients compared to healthy controls and patients with benign prostatic hyperplasia. Moreover, both proteins were detected in the cystic fluid and serum of patients with ovarian carcinomas [52]. The predominant detection of these proteins as part of the inflammatory response in pancreatic cancer rather than in tumour cells themselves means that their specificity for detecting cancer would necessitate the exclusion of other sources of inflammation. The S100A8/S100A9 complex has proven to be a useful diagnostic marker of inflammation in a variety of conditions, especially in non-infectious inflammatory diseases such as arthritis and chronic inflammatory lung and bowel disease [16, 18]. It has also been shown to predict the severity of glomerulonephritis [32]. We observed S100A8- and S100A9-positive inflammatory cells in tissues affected by chronic pancreatitis. Whether higher concentrations of S100A8 or S100A9 proteins will be found in blood/serum of patients with pancreatic cancer compared to patients with pancreatitis or healthy controls is a question that needs to be addressed directly. What seems clear, however, is that for either of these proteins to be useful positive markers of pancreatic cancer, other possible sources of inflammation, including pancreatitis, need to be excluded.

In conclusion, we have demonstrated overexpression of S100A9 and S100A8 in monocytes/immature macrophages in the stroma surrounding malignant pancreatic tumour cells. Furthermore, in a subset of these cells, the two proteins are co-expressed, and this relationship appears to be influenced by the Smad4 status of the corresponding tumour cells, providing evidence of tumour–host communication. Clearly, functional studies into the role of S100A8 and S100A9 in pancreatic cancer are required, but this study provides further novel evidence of the complex interaction between cancer and surrounding host cells.

This work was funded by grants from Cancer Research UK, The Royal College of Surgeons of England, The Medical Research Council, CORE and The Wellcome Trust. We are grateful to Professor John P. Neoptolemos for invaluable discussion and support throughout.

5 References

[1] Parkin, D. M., Bray, F. I., Devesa, S. S., *Eur. J. Cancer* 2001, 37 Suppl. *8*, S4–S6.

[2] Li, D., Xie, K., Wolff, R., Abbruzzese, J. L., *Lancet* 2004, *363*, 1049–1057.

[3] Sener, S. F., Fremgen, A., Menck, H. R., Winchester, D.P., *J. Am. Coll. Surg.* 1999, *189*, 1–7.

[4] Liotta, L. A., Kohn, E. C.*Nature* 2001, *411*, 375–379.

[5] Muerkoster, S., Wegehenkel, K., Arlt, A., Witt, M. *et al.*, *Cancer Res.* 2004, *64*, 1331–1337.

[6] Ohuchida, K., Mizumoto, K., Murakami, M., Qian, L. W, *et al.*, *Cancer Res.* 2004, *64*, 3215–3222.

[7] Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R. *et al.*, *Cancer Res.* 1999, *59*, 5002–5011.

[8] Bingle, L., Brown, N. J., Lewis, C. E., *J. Pathol.* 2002, *196*, 254–265.

[9] Koukourakis, M. I., Giatromanolaki, A., Brekken, R. A., Sivridis, E. *et al.*, *Cancer Res.* 2003, *63*, 5376–5380.

[10] Seymour, A. B., Hruban, R. H., Redston, M., Caldas, C. *et al.*, *Cancer Res.* 1994, *54*, 2761–2764.

[11] Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J. *et al.*, *Cancer Res.* 2002, *62*, 5351–5357.

[12] Iacobuzio-Donahue, C. A., Ryu, B., Hruban, R. H., Kern, S. E., Am. *J. Pathol.* 2002, *160*, 91–99.

[13] Koninger, J., Giese, T., di Mola, F. F., Wente, M. N. *et al.*, *Biochem. Biophys. Res. Commun.* 2004, *322*, 943–949.

[14] Shekouh, A. R., Thompson, C. C., Prime, W., Campbell, F. *et al.*, *Proteomics* 2003, *3*, 1988–2001.

[15] Odink, K., Cerletti, N., Bruggen, J., Clerc, R. G. *et al.*, *Nature* 1987, *330*, 80–82.

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[16] Nacken, W., Roth, J., Sorg, C., Kerkhoff, C., *Microsc. Res. Tech.* 2003, *60*, 569–580.

[17] Striz, I., Trebichavsky, I., *Physiol. Res.* 2004, *53*, 245–253.

[18] Foell, D., Frosch, M., Sorg, C., Roth, J., *Clin. Chim. Acta* 2004, *344*, 37–51.

[19] Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., Tessier, P. A., *J Immunol* 2003, *170*, 3233–3242.

[20] Ryckman, C., Gilbert, C., de Medicis, R., Lussier, A. *et al.*, *J. Leukoc. Biol.* 2004, *76*, 433–440.

[21] Crnogorac-Jurcevic, T., Missiaglia, E., Blaveri, E., Gangeswaran, R., *et al.*, *J. Pathol.* 2003, *201*, 63–74.

[22] Vimalachandran, D., Greenhalf, W., Thompson, C., Luttges, J. *et al.*, *Cancer Res.* 2005, *65*, 3218–3225.

[23] Dowen, S. E., Crnogorac-Jurcevic, T., Gangeswaran, R., Hansen, M. *et al.*, *Am. J. Pathol.* 2005, *166*, 81–92.

[24] Logsdon, C. D., Simeone, D. M., Binkley, C., Arumugam, T. *et al.*, *Cancer Res.* 2003, *63*, 2649–2657.

[25] Rosty, C., Ueki, T., Argani, P., Jansen, M. *et al.*, *Am. J. Pathol.* 2002, *160*, 45–50.

[26] Friess, H., Ding, J., Kleeff, J., Fenkell, L. *et al.*, *Cell. Mol. Life Sci.* 2003, *60*, 1180–1199.

[27] Han, H., Bearss, D. J., Browne, L. W., Calaluce, R. *et al.*, *Cancer Res.* 2002, *62*, 2890–2896.

[28] Wilentz, R. E., Iacobuzio-Donahue, C. A., Argani, P., McCarthy, D. M. *et al.*, *Cancer Res.* 2000, *60*, 2002–2006.

[29] Shen, J., Person, M. D., Zhu, J., Abbruzzese, J. L. *et al.*, *Cancer Res.* 2004, *64*, 9018–9026.

[30] Lu, Z., Hu, L., Evers, S., Chen, J., Shen, Y., *Proteomics* 2004, *4*, 3975–3988.

[31] Roth, J., Vogl, T., Sorg, C., Sunderkotter, C., *Trends Immunol.* 2003, *24*, 155–158.

[32] Frosch, M., Vogl, T., Waldherr, R., Sorg, C. *et al.*, *J. Leukoc. Biol.* 2004, *75*, 198–206.

[33] Manitz, M. P., Horst, B., Seeliger, S., Strey, A., *et al.*, *Mol. Cell. Biol.* 2003, *23*, 1034–1043.

[34] Vogl, T., Ludwig, S., Goebeler, M., Strey, A. *et al.*, *Blood* 2004, *104*, 4260–4268.

[35] Eue, I., Pietz, B., Storck, J., Klempt, M. *et al.*, *Int. Immunol.* 2000, *12*, 1593–1604.

[36] Hiratsuka, S., Watanabe, A., Aburatani, H., Maru, Y., *Nature Cell Biol.* 2006, *8*, 1369–1375.

[37] Ozawa, F., Friess, H., Tempia-Caliera, A., Kleeff, J. *et al.*, *Teratog. Carcinog. Mutagen.* 2001, *21*, 27–44.

[38] Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A. *et al.*, *Science* 1996, *271*, 350–353.

[39] Bardeesy, N., DePinho, R. A. *Nat. Rev. Cancer* 2002, *2*, 897–909.

[40] Rane, S. G., Lee, J. H., Lin, H. M., *Cytokine Growth Factor Rev.* 2006, *17*, 107–119.

[41] Schwarte-Waldhoff, I., Volpert, O. V., Bouck, N. P., Sipos, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 2000, *97*, 9624–9629.

[42] Duda, D. G., Sunamura, M., Lefter, L. P., Furukawa, T. *et al.*, *Oncogene* 2003, *22*, 6857–6864.

[43] Levy, L., Hill, C. S., *Mol. Cell. Biol.* 2005, *25*, 8108–8125.

[44] Massague, J., Blain, S. W., Lo, R. S., *Cell* 2000, *103*, 295–309.

[45] Rahimi, F., Hsu, K., Endoh, Y., Geczy, C. L., *FEBS J.* 2005, *272*, 2811–2827.

[46] Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R. *et al.*, *Clin. Exp. Immunol.* 1988, *72*, 510–515.

[47] Arai, K., Teratani, T., Kuruto-Niwa, R., Yamada, T. *et al.*, *Eur. J. Cancer* 2004, *40*, 1179–1187.

[48] Arai, K., Teratani, T., Nozawa, R., Yamada, T. *Oncol. Rep.* 2001, *8*, 591–596.

[49] Arai, K., Yamada, T., Nozawa, R., *Med. Oncol.* 2000, *17*, 183–188.

[50] Cross, S. S., Hamdy, F. C., Deloulme, J. C., Rehman, I., *Histopathology* 2005, *46*, 256–269.

[51] Hermani, A., Hess, J., De Servi, B., Medunjanin, S. *et al.*, *Clin. Cancer Res.* 2005, *11*, 5146–5152.

[52] Ott, H. W., Lindner, H., Sarg, B., Mueller-Holzner, E. *et al.*, *Cancer Res.* 2003, *63*, 7507–7514.

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Carcinogenesis vol.31 no.9 pp.1541–1551, 2010
doi:10.1093/carcin/bgq137
Advance Access publication July 9, 2010

Smad4 loss is associated with fewer S100A8-positive monocytes in colorectal tumors and attenuated response to S100A8 in colorectal and pancreatic cancer cells

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S100A8 and its dimerization partner S100A9 are emerging as important chemokines in cancer. We previously reported that Smad4-negative pancreatic tumors contain fewer stromal S100A8-positive monocytes than their Smad4-positive counterparts. Here, we studied S100A8/A9-expressing cells in colorectal tumors relating their presence to clinicopathological parameters and Smad4 status. Two-dimensional gel electrophoresis ($n = 12$) revealed variation in the levels of S100A8 protein in colorectal cancer tumors, whereas immunohistochemical analysis ($n = 313$) showed variation in the numbers of stromal S100A8-positive and S100A9-positive cells. Loss of Smad4 expression was observed in 42/304 (14%) colorectal tumors and was associated with reduced numbers of S100A8-positive ($P = 0.03$) but not S100A9-positive stromal cells ($P = 0.26$). High S100A9 cell counts were associated with large tumor sizes ($P = 0.0006$) and poor differentiation grade ($P = 0.036$). However, neither S100A8 nor S100A9 cell counts predicted poor survival, except for patients with Smad4-negative tumors ($P = 0.02$). To address the impact of environmental S100A8/A9 chemokines on tumor cells, we examined the effects of exogenously added S100A8 and S100A9 proteins on cellular migration and proliferation of colorectal and pancreatic cancer cells. S100A8 and S100A9 enhanced migration and proliferation in Smad4-positive and Smad4-negative cancer cells. However, transient depletion of Smad4 resulted in loss of responsiveness to exogenous S100A8, but not S100A9. S100A8 and S100A9 activated Smad4 signaling as evidenced by phosphorylation of Smad2/3; blockade of the receptor for the advanced glycation end products inhibited this response. In conclusion, Smad4 loss alters the tumor's interaction with stromal myeloid cells and the tumor cells' response to the stromal chemokine, S100A8.

Introduction

The complex interaction between tumor cells and surrounding non-malignant stromal host cells is increasingly understood to be a vital regulator of cancer growth and progression (1). Bone marrow-derived myeloid cells are an important component of the tumor microenvironment. Several bone marrow-derived myeloid cells, such as macrophages, Tie-2 expressing monocytes, neutrophils and mast cells have been shown to contribute to tumor angiogenesis (2). They have also been implicated in processes of tumor invasion and metastasis [for review see Joyce *et al.* (3)]. The presence of S100A8- and S100A9-positive myeloid cells in the pancreatic cancer microenvironment was previously characterized, revealing an association between the numbers of S100A8-expressing cells and the expression in tumor cells of the tumor suppressor protein Smad4 (4). The Smad4 gene is mutated at a high frequency in pancreatic and colon cancer and to a lesser extent in a variety of other cancers (5). The microenvironment of pancreatic tumors, which lacked expression of Smad4, was found to have significantly fewer S100A8-expressing cells compared with the microenvironment of Smad4-positive tumors (4). This finding suggested a phenotypic difference in the myeloid-derived infiltrate related to the Smad4 status of these tumors.

S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14) are low-molecular weight members of the S100 family of calcium-binding proteins, which are abundantly expressed in cells of the myeloid lineage, including monocytes and neutrophils and early-differentiation states of macrophages (6,7). They are secreted inflammatory chemoattractants that mediate further recruitment of inflammatory cells to sites of tissue damage (8) and have been implicated in a variety of chronic inflammatory conditions such as cystic fibrosis, rheumatoid arthritis, tuberculosis and transplant rejection (9,10). Recent attention has focused on the involvement of S100A8/A9 in cancer (11). These inflammatory proteins have been reported to promote tumorigenesis (12) and cause cancer metastasis by stimulating the migration of monocytes and tumor cells to metastatic sites (13,14).

In this study, stromal S100A8 and S100A9-expressing myeloid cells in colorectal tumors were thoroughly examined. The relationship between these myeloid cells and the Smad4 status of the colorectal cancers was determined as was the effect of exogenous S100A8 and S100A9 on Smad4-positive and Smad4-negative colorectal and pancreatic cancer cells.

Materials and methods

Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, protein identification and immunodetection

Proteins were extracted from frozen colectomy specimens, separated by two-dimensional electrophoresis and S100A8 identified as described previously (4,15). Colorectal cancer tissue microarrays were obtained from the Liverpool Tissue Bank, University of Liverpool and contained duplicate or triplicate cores from 313 independent specimens of adenocarcinoma. Fourteen cores of normal colon and 10 cores each of normal kidney, liver and testis were included as control tissue. Immunohistochemistry was undertaken as described previously (16) using polyclonal goat anti-S100A8, polyclonal rabbit anti-S100A9 or monoclonal anti-Smad4 (Clone-B8; Santa Cruz Biotechnology, Heidelberg, Germany) primary antibodies. S100A8 and S100A9 antibodies detect single bands in stimulated HL60 monocytic cells (supplementary Figure 1A is available at *Carcinogenesis* Online). Co-immunofluorescence was performed on duplicate formalin-fixed colorectal tumor sections using the following primary antibodies: a monoclonal mouse anti-S100A8 and polyclonal rabbit anti-S100A9 (Santa Cruz), monoclonal mouse anti-CD68 (Dako, Ely, Cambridgeshire, UK) and monoclonal mouse anti-CD14 (Novocastra, Newcastle, UK). Microarrays were scored by a specialist histopathologist (author B.A.) and an independent evaluator. The intensity of Smad4 staining (using a 0–3 scale) was recorded and a score of ≤ 0.5 (mean score of at least two cores per tumor) was classified as Smad4 negative. The number of stromal cells positive for S100A8 and S100A9 cells was counted (using ×40 magnification) for each tumor core and the mean number per tumor obtained by averaging the number of positive cells across all the tumor cores scored for that patient.

Abbreviations: MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); RAGE, receptor for the advanced glycation end products; siRNA, small interfering Ribonucleic acid.

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Generation of recombinant S100A8 and S100A9 proteins

pGEX4T-1 plasmids encoding S100A8-GST and S100A9-GST (13) were kindly provided by Y.Maru, Tokyo Women's Medical University School of Medicine, Japan. Proteins were expressed in *Escherichia coli* following induction with isopropyl-b-D-thiogalactopyranoside (VWR international, Lutterworth, Leicestershire, UK) and purified with glutathione sepharose beads (GE Healthcare, Amersham, Buckinghamshire, UK). The quantity of recombinant S100A8-GST and S100A9-GST was determined by performing one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis against serial dilutions of a reference standard of bovine serum albumin (1–15 μg/lane) followed by densitometric evaluation of the Coomassie-stained gels (GS-800 scanner; Bio-Rad, Bath, UK) using QuantityOne software (Bio-Rad). All experiments using fusion proteins were performed with at least two independent batches of each protein.

Cell lines and western blotting

The rectal cell line SW837 was purchased from the European Collection of Cell Cultures in March 2008. The colonic cell line SW480 and the pancreatic adenocarcinoma cell line Panc-1 were obtained from American Type Culture Collection in 1999. Neomycin-resistant clonal derivatives of Smad4-deficient SW480 cells, stably reexpressing Smad4 (SWD20) and a negative control transfectant (SWK3), described previously (17,18) were maintained in medium containing geneticin (0.2 mg/ml;Gibco, Paisley, UK). All cell lines were last authenticated in October 2009 using short tandem repeat profiling against the international reference standard for cell line.

Western blot analysis was performed as described previously (16) using mouse anti-Smad4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Smad2, anti-phospho-Smad3, anti-phospho-Smad1/5/8 antibodies (Cell Signaling Technology, Massachusetts, MA) and mouse anti-β-actin monoclonal antibody (Sigma–Aldrich, Gillingham, Dorset, UK).

In vitro cell migration assay

Cells were plated (5×10^4 for SW837/SW480/SWD20/SWK3 and 5×10^3 for Panc-1) in medium containing 1% fetal bovine serum for Boyden Chamber assays, conducted over 18 h, as described previously (16). Recombinant proteins, S100A8-GST, S100A9-GST or GST in medium containing 1% fetal bovine serum were added to the lower transwell chambers. Migrated cells were stained and counted (16), $n = 2$ inserts per treatment, and the average number of migrated cells were calculated. Experiments were performed at least three times.

Cell proliferation assay

Cells (3×10^3/200 μl medium, supplemented with 1% fetal bovine serum) were plated in wells of 96-well plates and recombinant S100A8-GST, S100A9-GST or GST added. Proliferation was assessed at 24, 48 and 72 h using the EZ4U non-radioactive cell proliferation assay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. Absorbance readings at 450 nm were taken at 4 h following incubation with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagents. Experiments were performed at least three times and five wells utilized for each treatment.

Cellular phospho-Smad expression and blocking of receptor of advanced glycation end products with specific blocking antibody

Cells were cultured for 48 h in serum-free medium supplemented with bovine serum albumin (10 μg/ml; Sigma–Aldrich) and human insulin (5 μg/ml; Sigma–Aldrich) and then treated with S100A8-GST, S100A9-GST, GST or transforming growth factor-β (10 ng/ml; PeproTech EC Ltd, London, UK) for 1 h, followed by cell collection for western blotting as described above. For receptor of advanced glycation end products (RAGE) blocking, cells were treated with RAGE-blocking antibody (40 or 80 μg/ml; R&D Systems, Abingdon, UK) for 1 h before the addition of recombinant proteins.

Transient Smad4 knockdown

Small interfering Ribonucleic acid (siRNA) experiments were performed as described previously (16). Smad4-targeting siRNAs, GUGUGCAGUUGGAAUGUAA (Smad4 siRNA1) and GUACAGAGUUACUACUUAG (Smad4 siRNA2) were purchased from Dharmacon (Chicago, IL). Two non-targeting control siRNAs were used, control 1 (siControl non-targeting siRNA 1 from Dharmacon) and control 2 (GGACGCAUCCUUCUUAA, a gift from M.Boyd, University of Liverpool, UK). Smad4 levels diminished between 48 and 72 h post-transfection with Smad4-targeting siRNAs and remained low out to 120 h (data not shown).

Cellular immunofluorescence

Cells were fixed with 4% formaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. Phospho-Smad2/3 was detected with anti-phospho-Smad2/3 antibody (Cell Signaling Technology) and visualized with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories West Grove, PA). Actin filaments were labeled with phalloidin (Invitrogen, Renfrew, UK).

Statistical analysis

Comparisons between two groups were made using nonparametric continuity corrected chi-square test or chi-squared test, when more than two groups were analyzed. Continuous variables were compared using the Mann–Whitney *U*-test or the Wilcoxon signed-rank test. To evaluate the effect of stromal S100A8 or S100A9 cells on patient survival, life tables were constructed and Kaplan–Meier curves plotted. Overall survival was measured from date of initial surgery to date of death, counting death from any cause as the end point or the last date of information as the end point if no event was documented. To analyze data from 'motility' or 'proliferation' experiments, continuous variables were compared using the Student's *t*-test and were expressed as mean. All analyses were performed using Statview Version 5.01 (SAS Institute Cary, North Carolina). A *P*-value of <0.05 was considered significant.

Results

Variable S100A8 and S100A9 levels in colorectal cancer microenvironments

Two-dimensional gels displaying proteins extracted from 12 individual undissected colorectal tumors revealed variation (Figure 1A–C) in the intensity of a spot (arrowed in Figure 1A and B) that was suspected, on the basis of its gel location, to contain S100A8. Protein recovered from this spot was trypsin digested and analyzed by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (Figure 1D). This resulted in the detection of five peptides, providing sequence coverage of 37.6% and confirmed the identification of S100A8 (National Centre for Biotechnology Information accession no. CAG28602). To establish the cellular basis for the variability of S100A8, immunohistochemistry of formalin-fixed colorectal adenocarcinomas was undertaken, for the detection of S100A8 and its dimerization partner S100A9. This revealed the expression of both proteins in cells scattered throughout the tumor stroma [Figure 2A(i–iv)]. While S100A9 expression was not detected in cancer cells, a very low intensity of cytoplasmic S100A8 staining was observed in tumor cells of virtually all cases and was not subjected to analysis. Co-immunofluorescence indicated extensive colocalization of S100A8 and S100A9 in stromal cells [Figure 2B(i)]. Furthermore, co localization of S100A8 or S100A9 was observed in some cells with the monocyte marker CD14 [Figure 2B(ii and iii), respectively] but not the macrophage marker CD68 [Figure 2B (iv and v), respectively).

The number of S100A8-positive or S100A9-positive cells in each of 313 patient tumors (mean of at least two Tissue Microarray cores per tumor case) was determined. Numbers ranged from 0 (4.5% of cases) to 288 and from 0 (0.65% of cases) to 882 for S100A8 and S100A9, respectively. The median number of stromal S100A8-expressing cells was 23 [Inter-quartile range (IQR) 6–70], whereas the median number of stromal S100A9-expressing cells was 65 (IQR 27–126) with 284/308 (92%) tumors showing more S100A9 than S100A8-expressing cells in the stroma. Although tumors generally contained fewer S100A8-positive than S100A9-positive cells ($P < 0.0001$, Wilcoxon signed-rank test), there was a strong positive relationship between S100A8 and S100A9 counts ($n = 302$ independent tumor cases; $R^2 = 0.76$, $P < 0.0001$). This is entirely consistent with our observation that S100A8 colocalized with S100A9. The median S100A9:S100A8 ratio was 2.2 (IQR 1.3–4.4). To examine whether a relationship existed between the numbers of stromal S100A8-positive or S100A9-positive cells and the Smad4 tumor status, the expression of Smad4 protein was determined by immunohistochemistry [Figure 2A (v and vi)]. Forty-two of 304 patients (14%) were categorized as Smad4 negative based on mean cytoplasmic intensity scores of ≤ 0.5. The remaining 262 of 304 patients (86%) were categorized as Smad4 positive. Loss of Smad4 expression in primary colorectal tumor cells was associated with a significantly lower median count of S100A8-positive stromal cells (14, IQR 5–37) compared with a median S100A8-positive cell count of 25 (IQR 6–76) in the Smad4-positive group [Figure 2C (i)], $P = 0.03$, Mann–Whitney *U*-test). A similar relationship was not observed between

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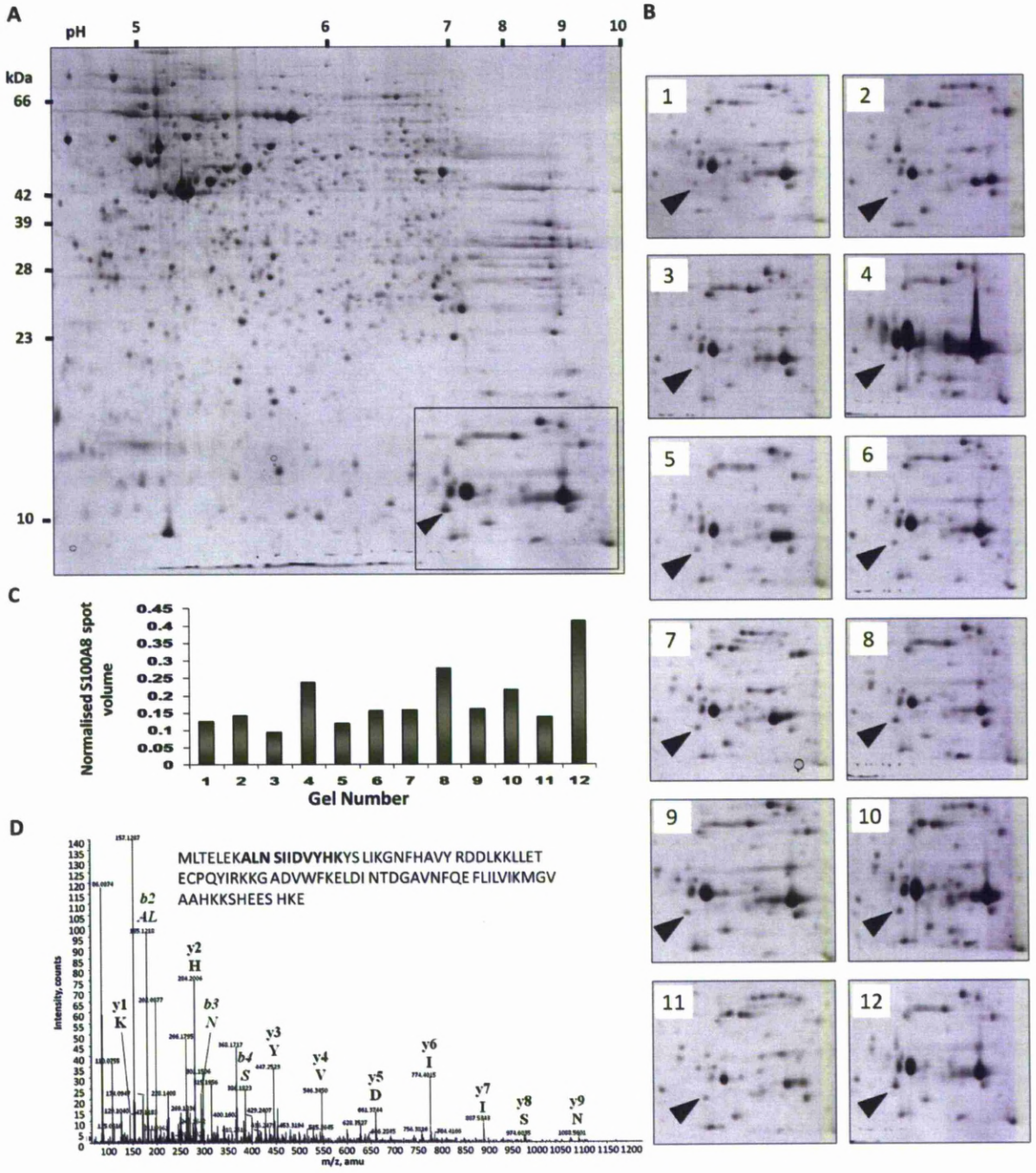

Fig. 1. (**A**) Colloidal Coomassie Blue-stained two-dimensional gel image of a colorectal cancer lysate, with the S100A8-containing spot arrowed. (**B**) Insets from 12 independent colorectal tumor gels showing different intensities in the S100A8 protein spot. (**C**) Normalized S100A8 levels (S100A8 spot intensity/total spots intensity). (**D**) Mass Spectrometry/Mass Spectrometry spectrum of the peptide ALNSIIDVYHK from S100A8.

tumor cell Smad4 expression and S100A9-positive stromal monocytes [Figure 2C (ii)], $P = 0.26$).

Relationship between S100A8/A9-positive stromal cells and clinicopathological characteristics

To examine for associations between stromal S100A8-positive cells and patient clinicopathological parameters, patients were categorized as having mean S100A8-positive cell counts that were low ($\leq$ median of 23) or high (>23). Similarly, for S100A9, patients were categorized as having low ($\leq$ median of 65) or high (>65) cell counts. The degree of stromal S100A8/A9-positive infiltration showed no correlation with the parameters of age at surgery, gender, site of tumor, depth of tumor invasion or nodal metastases (Table I). However, high cell S100A8 and S100A9 counts were

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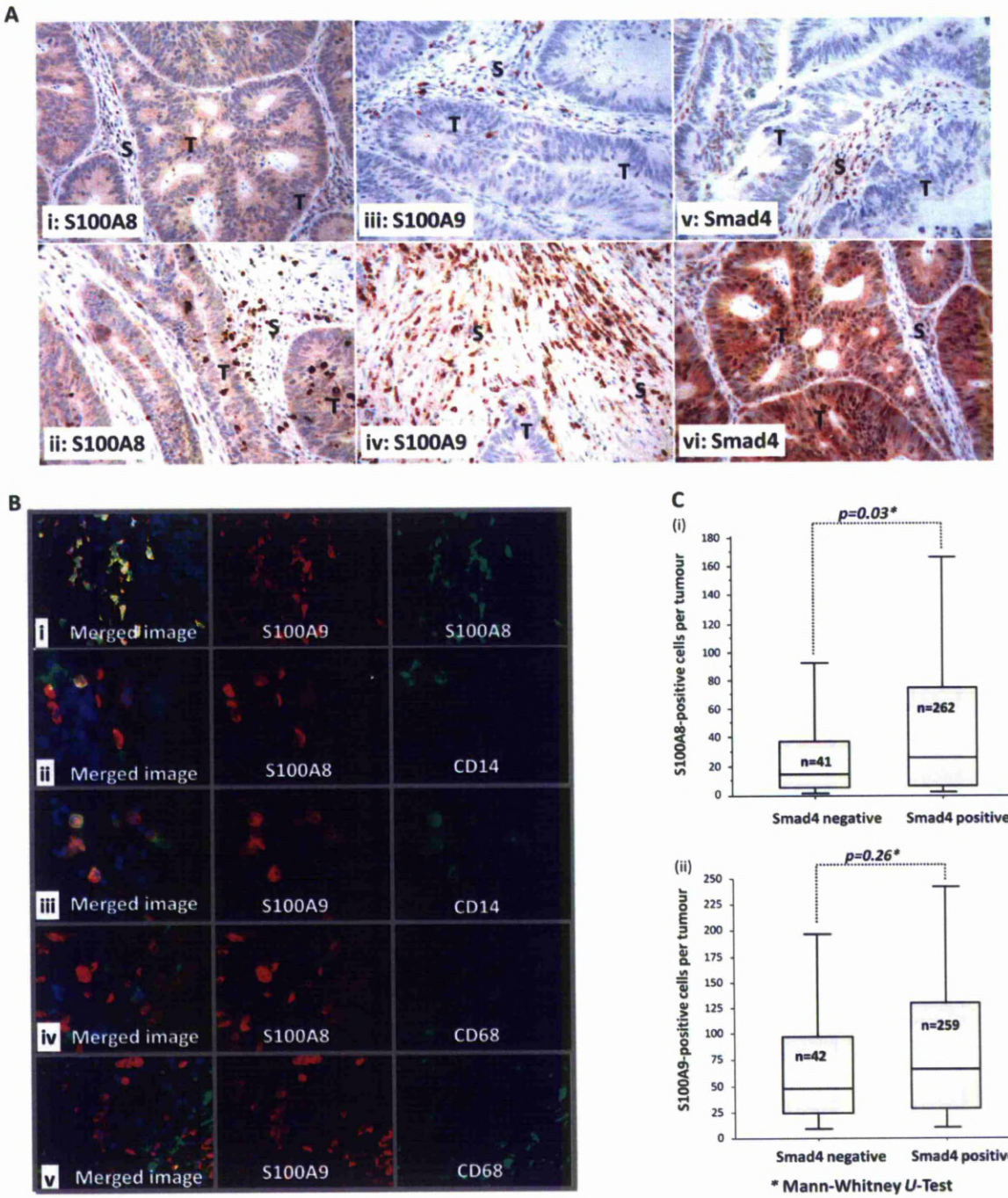

Fig. 2. Colorectal cancer tissue illustrating a low [**A**(i and iii)], and a high [A(ii and iv)] infiltrate of S100A8- and S100A9-positive cells, respectively; the absence and presence, respectively, of Smad4 staining [A(v and vi)]; colocalization of S100A9 and S100A8 [B(i)], S100A8 or S100A9 and CD14 [**B**(ii and iii)]; lack of colocalization of S100A8 or S100A9 and CD68 [B (iv and v)]. The mean numbers of S100A8-positive [C(i)] or S100A9-positive cells [**C**(ii)] per tumor were plotted for Smad4-negative and Smad4-positive tumors.

associated with larger tumor size ($P = 0.01$ and 0.0006, respectively). These proteins are co-expressed to a high degree. Nevertheless, we wished to determine whether either protein was independently associated with tumor size. We therefore performed logistic regression analyses. Both high S100A8 and high S100A9 cell counts were associated with large tumor size on univariate analysis ($P = 0.01$ and 0.0007, respectively; supplementary Table 1 is available at *Carcinogenesis* Online). However, on multivariate analysis, only high S100A9 cell counts remained independently associated with large tumor sizes ($P = 0.02$). High S100A9 cell counts were also associated with poor differentiation grade ($P = 0.036$).

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Table I. Clinicopathological characteristics and correlation with S100A8 and S100A9 expression

	All cases, $n = 313$ (%)	S100A8-positive cells, $n = 309$		P value[a]	S100A9-positive cells, $n = 306$		P value[a]
		Low $\leq$ 23 cells (%), $n = 156$	High $>$ 23 cells (%), $n = 153$		Low $\leq$ 65 cells (%), $n = 159$	High $>$ 65 cells (%), $n = 147$	
Age 70 (IQR 62–76 years)							
Young (<median)	155 (49)	75 (48)	80 (52)	0.459	75 (47)	78 (53)	0.303
Old (>median)	158 (51)	81 (52)	73 (48)		84 (53)	69 (47)	
Gender							
Male	188 (60)	93 (60)	92 (60)	0.926	97 (61)	85 (58)	0.570
Female	125 (40)	63 (40)	61 (40)		62 (39)	62 (42)	
Site of tumor							
Colon	188 (60)	89 (57)	96 (63)	0.307	94 (59)	90 (61)	0.707
Rectum	125 (40)	67 (43)	57 (47)		65 (41)	57 (39)	
Size 50 (IQR 38–60 mm)							
Small-medium (<60 mm)	214 (69)	116 (74)	94 (61)	**0.014**	123 (77)	87 (59)	**0.0006**
Large (≥60 mm)	99 (31)	40 (26)	59 (39)		36 (23)	60 (41)	
Differentiation grade							
Well	5 (2)	2 (1)	2 (1)	0.819	2 (1)	3 (2)	**0.036**
Moderate	283 (90)	140 (90)	140 (92)		150 (94)	127 (86)	
Poor	21 (7)	12 (8)	9 (6)		5 (4)	15 (11)	
Uncategorized/DNA	4 (1)	2 (1)	2 (1)		2 (1)	2 (1)	
Excision margin							
Clear	270 (86)	130 (83)	138 (90)	0.055	134 (84)	130 (88)	0.199
Involved	39 (13)	25 (16)	12 (8)		24 (15)	14 (10)	
Uncategorized/DNA	4 (1)	1 (1)	3 (2)		1 (1)	3 (2)	
T-stage							
T1	13 (4)	8 (5)	5 (3)	0.766	8 (5)	5 (3)	0.356
T2	47 (15)	22 (14)	25 (16)		21 (13)	24 (16)	
T3	206 (66)	104 (67)	101 (66)		110 (69)	92 (63)	
T4	44 (14)	19 (12)	22 (15)		18 (12)	25 (17)	
Uncategorized/DNA	3 (1)	3 (2)	0		2 (1)	1 (1)	
N-stage							
N0	177 (56)	82 (53)	93 (61)	0.446	90 (57)	82 (56)	0.332
N1	71 (23)	38 (24)	32 (21)		40 (25)	30 (20)	
N2	62 (20)	33 (21)	28 (18)		27 (17)	34 (23)	
Uncategorized/DNA	3 (1)	3 (2)	0		2 (1)	1 (1)	
AJCC/UICC stage groupings							
I	39 (13)	22 (14)	17 (11)	0.319	21 (13)	17 (12)	0.860
II	136 (43)	60 (38)	74 (48)		68 (43)	65 (44)	
III	132 (42)	70 (45)	60 (39)		66 (41)	63 (43)	
Uncategorized/DNA	6 (2)	4 (3)	2 (2)		4 (3)	2 (1)	
Chemoradiotherapy							
Neoadjuvant only	35 (11)	21 (13)	14 (9)	0.630	19 (12)	15 (10)	0.724
Adjuvant only	77 (25)	37 (24)	40 (26)		37 (23)	40 (27)	
Neoadjuvant and adjuvant	12 (4)	6 (4)	5 (3)		6 (4)	4 (3)	
No chemoradiotherapy	188 (60)	92 (59)	93 (61)		97 (61)	87 (59)	
DNA	1 (0.3)	0 (0)	1 (1)		0 (0)	1 (1)	

AJCC/UICC, American Joint Committee on Cancer/International Union Against Cancer; DNA, data not available.
[a]Chi-squared test.

Neither S100A8-positive nor S100A9-positive stromal cell counts were associated with patient survival [Figure 3A (i and ii)]. However, for the smaller cohort of patients exhibiting loss of Smad4 expression, high S100A8 or S100A9 cell counts predicted poor 3 year survival [Figure 3B (i and ii)]. The number of Smad4-negative tumor patients in the study was too small to determine whether either of the proteins was independently associated with poor outcome. No survival difference was observed for either high S100A8 or S100A9 counts at 60 months in this Smad4-negative cohort. For patients with Smad4-positive tumors, neither S100A8-positive nor S100A9-positive counts predicted overall short- or long-term survival [Figure 3C (i and ii)].

The effects of Smad4 status on S100A8- and S100A9-induced migration activity

S100A8 and S100A9 are secreted chemokines, which could affect tumor cells in their vicinity. We next investigated their chemoattractive functions on tumor cells *in vitro*. The addition of purified S100A8-GST and S100A9-GST fusion proteins (see supplementary Figure 1B, available at *Carcinogenesis* Online) at concentrations of either 0.4 or 2 μg/ml significantly increased the migration activity of the rectal cancer cell line SW837, the colon cancer cell line SW480 and the pancreatic cancer cell line Panc-1 (Figure 4A) ($P < 0.001$) compared with the addition of purified GST control protein. The addition of both S100A8-GST and S100A9-GST proteins simultaneously did not significantly increase the response over proteins added individually (see supplementary Figure S2, available at *Carcinogenesis* Online) and may be due to failure of the recombinant proteins to interact correctly.

Both Panc-1 and SW837 cells express Smad4 (see inset Figure 4A). To determine whether the Smad4 status of cells influenced their response to S100A8 or S100A9, we depleted Smad4 in these cell lines and examined their migration activity in response to S100A8 and S100A9. An ~50% decrease in basal migration in Panc-1 cells compared with control siRNA-treated cells was observed (Figure 4B), consistent with previously published data (19). Smad4 knockdown totally suppressed migration responses toward S100A8. In contrast, S100A9-treated cells retained an ~2-fold increase in migration after

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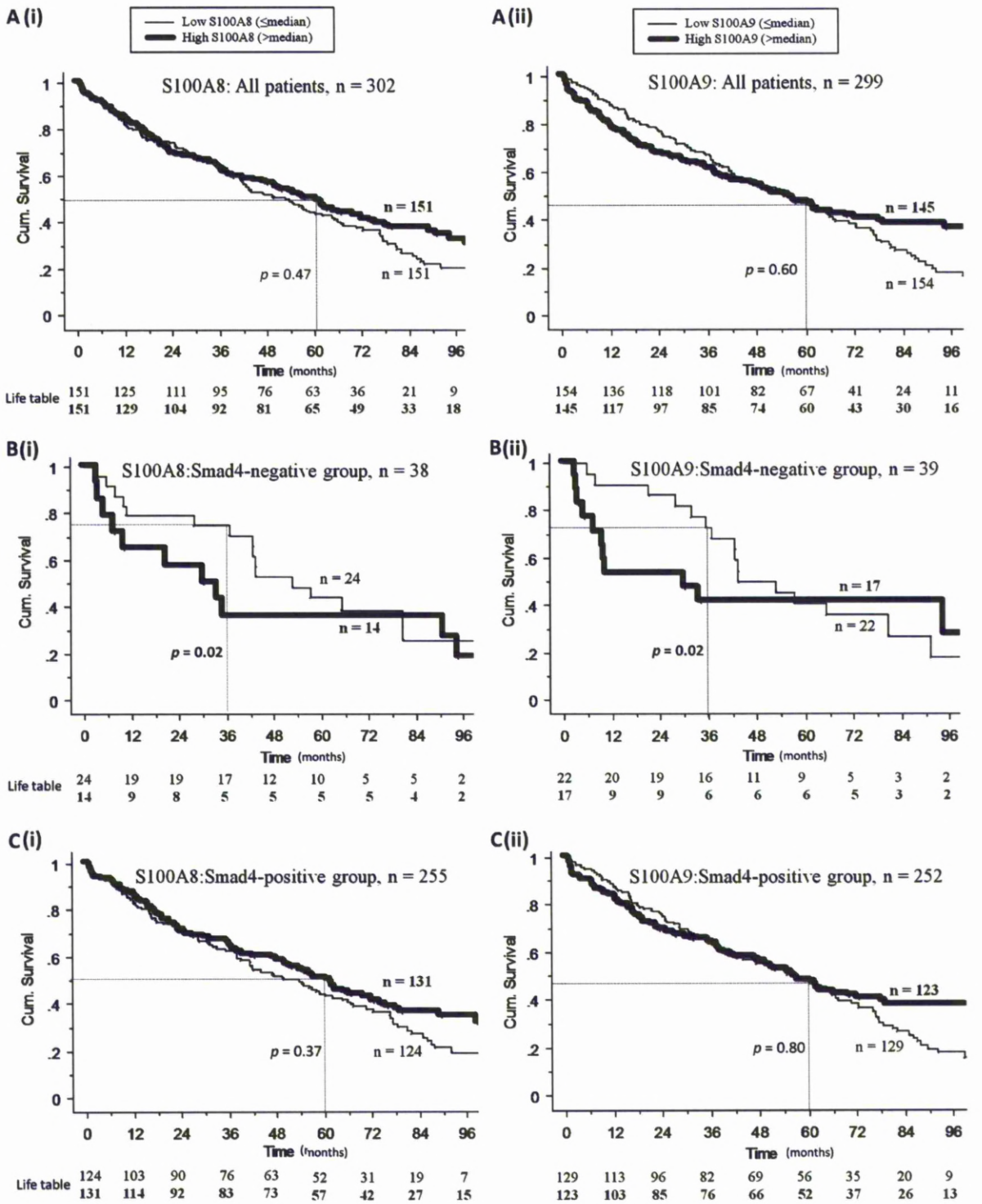

Fig. 3. Kaplan–Meier graphical analysis showing (**A**) no survival difference based on S100A8 [A(i)] or S100A9 [A(ii)] cell counts in all patients, (**B**) a survival difference in patients with Smad4-negative tumors based on S100A8 [B(i)] or S100A9 [B(ii)] cells counts and (**C**) no survival difference in patients with Smad4-positive tumors based on S100A8 [C(i)] or S100A9 [C(ii)] cell counts.

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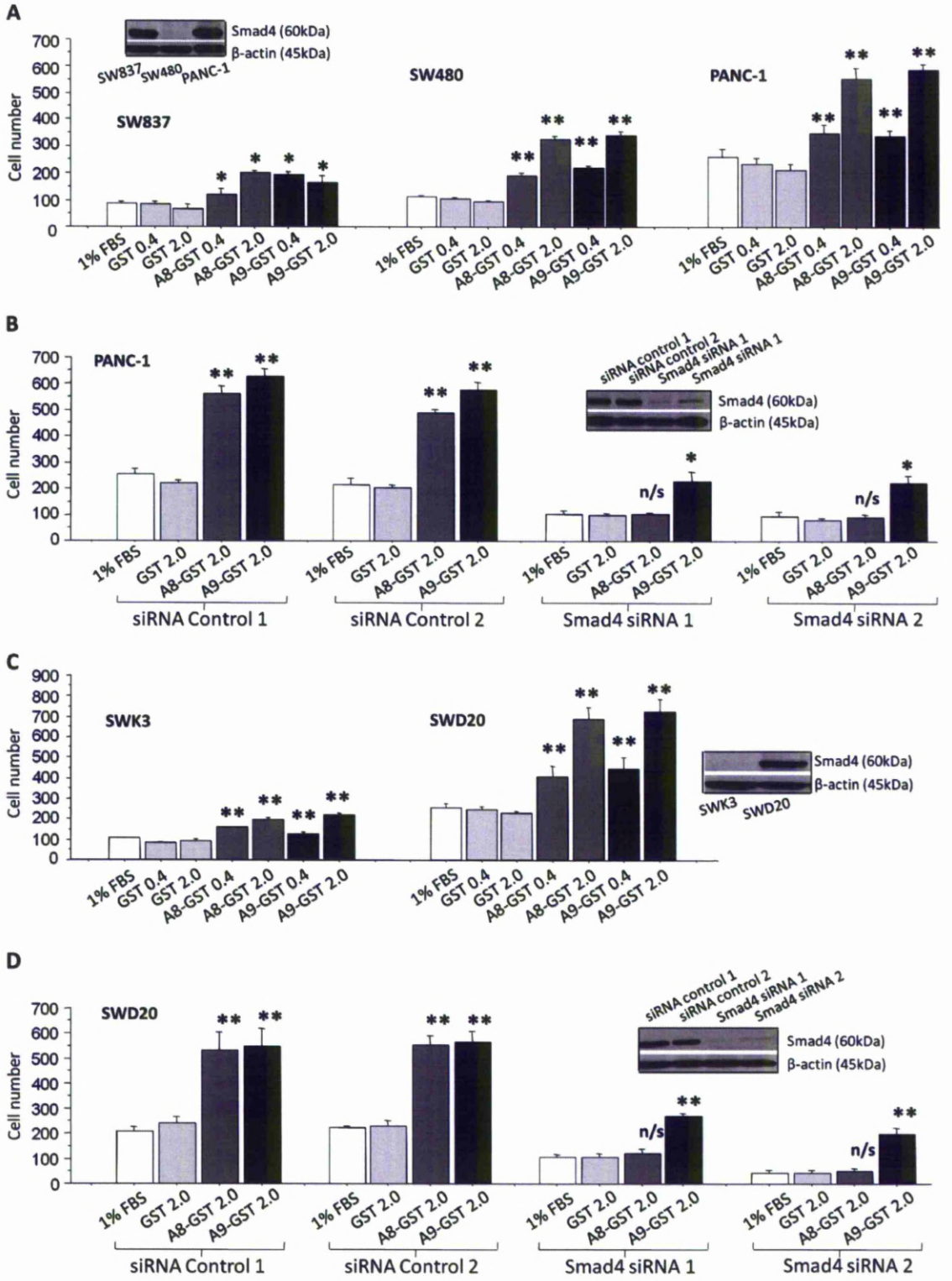

Fig. 4. Migration activities of indicated cell lines treated with S100A8-GST (A8-GST), S100A9-GST (A9-GST), GST at concentrations of 0.4 or 2.0 μg/ml, in medium containing 1% fetal bovine serum (FBS) (**A**) or Smad4-depleted Panc-1 cells (**B**) or clonal derivative of SW480 cells that stably reexpresses Smad4 (SWD20) and its Smad4-negative control (SWK3) (**C**) or Smad4-depleted SWD20 (**D**). $^{**}P < 0.001$, $^{*}P < 0.05$. The Smad4 status of cells is shown as insets on western blots.

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Smad4 knockdown. Smad4 knockdown caused an almost total loss of basal and chemokine-induced migration of the less motile SW837 cells (supplementary Figure 3 is available at *Carcinogenesis* Online).

SW480 cells do not express Smad4 (see inset Figure 4A). Neomycin-resistant clonal derivatives of SW480 cells, stably reexpressing Smad4 (SWD20) and negative control transfectants (SWK3) (17,18) were used to determine whether restoration of Smad4 would alter responsiveness to S100A8 or S100A9. We found that both the Smad4-negative and Smad4-positive subclones had enhanced migration following incubation with S100A8-GST or S100A9-GST (Figure 4C). However, transient siRNA-mediated depletion of Smad4 from SWD20 cells resulted in a reduction in motility and a loss of responsiveness to S100A8-GST, but not S100A9-GST (Figure 4D). This was similar to the result obtained with Panc-1 cells (Figure 4B).

The effects of Smad4 status on S100A8- and S100A9-induced proliferation

Similarly, we sought to examine the effects of S100A8 and S100A9 on tumor cell proliferation and explore whether Smad4 status was important in this context. Incubation of SW837 and SW480 cells with 2 μg/ml of S100A8-GST or S100A9-GST resulted in modest, though statistically significant increases in MTS readings (Figure 5A). No increase was observed using 0.4 μg/ml recombinant proteins (data not shown) and Panc1 cells did not show an increased MTS response at all (supplementary Figure 4A, available at *Carcinogenesis* Online). In the case of SW837 cells, the simultaneous addition of S100A8-GST and S100A9-GST proteins at 2 μg/ml significantly improved the response over proteins added individually (see supplementary Figure 4B, available at *Carcinogenesis* Online), although this effect was not observed with SW480 or Panc-1 cells (supplementary Figure 4C and D is available at *Carcinogenesis* Online).

The Smad4-negative SW480 subclone, SWK3 showed enhanced proliferation in response to 2 μg/ml S100A9-GST (Figure 5B), whereas the Smad4 reexpressing subclone, SWD20, responded to both S100A8-GST and S100A9-GST, although the response to S100A9-GST was greater (Figure 5B). Following depletion of Smad4 from SWD20 cells, responsiveness to S100A8-GST was lost (Figure 5C). S100A9-GST continued to induce proliferation, although the extent of proliferation reached was not as great as that observed in control siRNA-treated cells (Figure 5C). Depletion of Smad4 from Panc-1 cells gave a similar result (supplementary Figure 5 is available at *Carcinogenesis* Online) although the increase in MTS readings in response to S100A9 was very small.

The effects of exogenous S100A8 and S100A9 on Smad4 signaling and RAGE

Our results indicated that the effects of exogenous S100A8 and S100A9 were influenced to an extent by the presence of Smad4 in tumor cells. To examine whether these proteins could signal via Smad4, cells were incubated for 1 h with 2 μg/ml of S100A8-GST or S100A9-GST and the activation of members of the Smad pathway examined. Both recombinant S100 proteins alone, but not GST, induced increased levels of phospho-Smad2, phospho-Smad3, but not phospho-Smad1/5/8 in Panc-1 cells [Figure 5D (i)]. Furthermore, phospho-Smad2/3 was shown to accumulate in the nucleus following incubation with S100A8-GST or S100A9-GST, but not GST (supplementary Figure 6 is available at *Carcinogenesis* Online). Similar results were obtained with SWD20 cells (data not shown). S100A8/A9 heterodimers are known to stimulate cells through binding to the cellular receptor RAGE (20,21), although it is unclear whether the individual proteins are ligands for this receptor. RAGE expression was observed in the cell lines used in this study [Figure 5D (ii)]. To determine whether S100A8 and S100A9 were activating the Smad pathway through RAGE, we used a RAGE-blocking antibody at two concentrations, 40 and 80 μg/ml. When Panc-1 cells were pretreated with the RAGE-blocking antibody, a dose-dependent reduction of phopho-Smad2 and phospho-Smad3 levels was observed in response to S100A8-GST or S100A9-GST stimulation [Figure 5D (iii)].

Discussion

The inflammatory chemoattractants, S100A8 and S100A9, apart from serving as markers of phagocytes (intracellular S100A8/A9) or as biomarkers of inflammatory disease conditions (secreted extracellular S100A8/A9), are now recognized to play important roles themselves in the pathogenesis of inflammatory disorders and more recently in cancer (11). The presence of S100A8- and S100A9-positive cells has been reported previously in smaller studies of colorectal cancer (12,22). Here, we provide a comprehensive analysis, in which we quantified the numbers of S100A8/A9 cells infiltrating the environment of colorectal tumors. We also noted expression of S100A8/A9 proteins in neutrophils localized in the tumor vasculature, although this cell type was not studied further. S100A8 and S100A9 proteins were largely co-expressed in the same cells, and the majority of tumors (92%) contained more S100A9-positive cells than S100A8-positive cells, which may reflect the differentiation state of the myeloid cells expressing these proteins (23). There was overlap in the expression of S100A8 and S100A9 and the monocyte/macrophage marker CD14, although the S100A8/A9-positive cell population did not express the mature macrophage marker CD68, indicating that the S100A8/A9 population may be at an early stage of monocyte/macrophage maturation.

Relatively little is known about tumor-associated monocytes in colorectal cancer, although $CD68^+$ tumor-associated macrophages have been analyzed in detail. High infiltrates of $CD68^+$ tumor-associated macrophages in colorectal tumors were shown to correlate with absence of vascular and lymph node invasion (24) and improved survival (25,26). No evidence was found for an association between the numbers of S100A8/A9-positive cells and either depth of tumor invasion or nodal metastases. High S100A9 infiltrate, however, was associated with poor differentiation grade and correlated strongly with larger tumor size. In contrast to the reported links between high $CD68^+$ infiltrate and improved outcome, when the entire cohort of ~300 patients was examined as a whole, no association was found between S100A8/A9 levels and survival. Nonetheless, the short-term survival of patients with Smad4-negative tumors and high A8/A9 infiltrate was significantly poorer than Smad4-negative patients with low A8/A9 infiltrate. This suggests that Smad4-negative tumors have a shorter time to recurrence or metastases in the presence of high stromal S100A8- or S100A9-expressing monocytes. Mutations or inactivation of Smad4 in colorectal carcinoma coincide with progression to metastatic disease; the highest percentages of inactivation (>30%) are observed in patients with distant organ metastasis (27). In our study, only non-metastatic tumors with stages I–III disease were included, which accounts for the lower frequency of Smad4 loss (14%) in our cohort.

One of the main aims of our study was to determine if the extent of the S100A8/A9-positive infiltrate was influenced by the Smad4 status of the colorectal tumors under examination. The motivation to examine this stemmed from the previous observation that Smad4-negative pancreatic tumors contained fewer S100A8-positive stromal cells than Smad4-negative pancreatic tumors (4). Furthermore, using a mouse model of colon cancer, Kitamura *et al.* (28) reported that Smad4-negative tumors recruited a specific type of myeloid cell, which promoted invasion through cross talk with tumor cells. We observed a distinctive reduction in the numbers of S100A8-positive, but not S100A9-positive stromal cells in Smad4-negative colorectal tumors, resulting in a change in the relative levels of S100A8 to S100A9. The S100A8/A9 myeloid cell population that we have studied does not appear to be identical to that observed in the mouse study of Kitamura *et al.* (28) because the mice cells lacked expression of CD14. However, our observation that the loss of Smad4 is accompanied by a change to the phenotype of the myeloid infiltrate is consistent with that of Kitamura *et al.* and provides important evidence in human colorectal cancer that the phenotype of the myeloid infiltrate is influenced by the Smad4 status of the tumor.

Hiratsuka *et al.* (13) showed that S100A8/A9 were powerful chemoattractants whose tumor-induced presence in the lungs of tumor-bearing mice could stimulate the migration of Lewis Lung carcinoma

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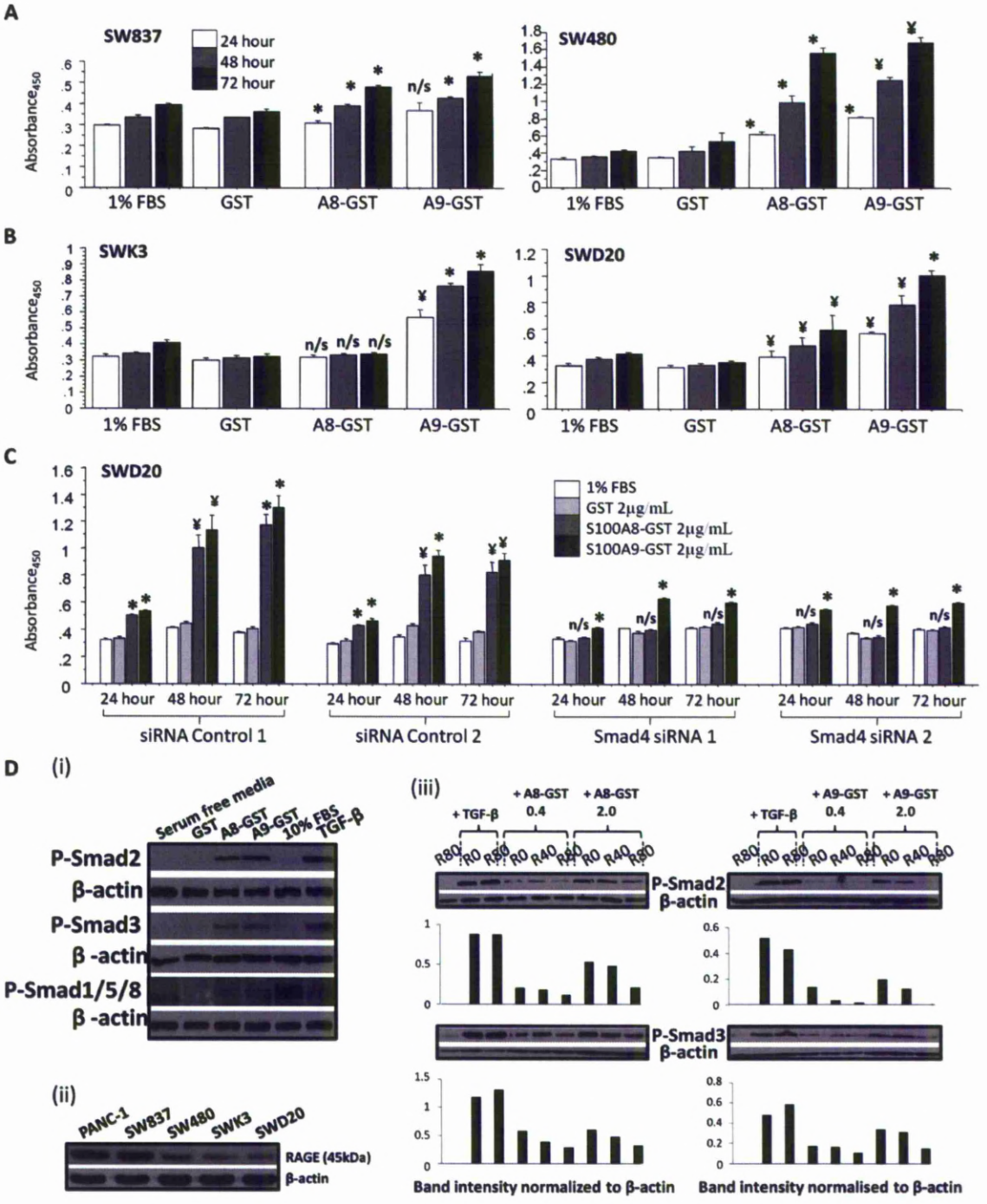

Fig. 5. MTS readings of indicated cells in response to S100A8-GST (A8-GST), S100A9-GST (A9-GST) or GST at a concentration of 2.0 μg/ml (**A**) or SW480 derivatives (**B**) or Smad4-depleted SWD20 (**C**). $^{*}P < 0.01$, $¥P < 0.05$. Western detection of phospho-Smad2, phospho-Smad3 and phospho-Smad1/5/8 levels [**D**(i)] following treatment of Panc-1 cells with 2 μg/ml of S100A8-GST (A8-GST), S100A9-GST (A9-GST) or GST. Transforming growth factor (TGF-β) (10 ng/ml) and 10% fetal bovine serum (FBS) were the positive control for phospho-Smad2/3 and phospho-Smad1/5/8 activation, respectively. Western detection of RAGE expression [**D**(ii)]. Western detection and densitometric representation of the levels of phospho-Smad2 and phospho-Smad3, which were reduced in Panc-1 cells pretreated with RAGE-blocking antibody (R) (40 and 80 μg/ml) before the addition of 0.4 or 2 μg/ml of S100A8-GST or S100A9-GST [D(iii)].

cells and B16 melanoma cells to that organ, where they formed secondary tumors. We sought to determine whether the response of tumor cells to S100A8/A9 was potentially influenced by their Smad4 status. Both S100A8 and S100A9 were highly chemotactic for rectal, colon and pancreatic cancer cell lines, regardless of whether the cells expressed Smad4 or not. In Smad4-expressing cells, such as Panc-1 cells

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or the SWD20 cells, where Smad4 expression has been stably restored, transient depletion of Smad4 expression was accompanied by a loss of responsiveness to S100A8-induced migration activity but not that of S100A9, suggesting that S100A8 elicits its response through a Smad4-dependent pathway. Transient Smad4 depletion, such as we undertook in our siRNA experiments, may not allow sufficient time for cells to adapt and to allow S100A8 to signal in a Smad4-independent manner. Our observation of increased levels of phosphor-Smad2 and phospho-Smad3 in response to cell treatment with S100A8 and S100A9 provide supporting evidence that these proteins can activate the Smad4 signaling pathway.

S100A8/A9-induced proliferation of the cancer cell lines studied was also observed, although at concentrations higher that those required to induce migration activity. S100A8/A9 may contribute to apoptosis (11), however, the apoptotic role of these proteins was not investigated in this study. Ghavami *et al.* (21) reported that S100A8 and S100A9 proteins promoted growth of human breast cancer and neuroblastoma cells and confirmed that these proteins activate the multiligand receptor, RAGE, triggering the mitogen-activated protein kinase signaling pathway. The mitogenic effects of S100A8/A9, coupled with our observation that high levels of A8/A9 infiltrate were associated with larger tumors suggest that the proteins may contribute to the growth of these tumors. Interestingly, while the Smad4-restored SWD20 cells showed increased proliferation in response to S100A8-GST and S100A9-GST, their Smad4-negative clonal counterpart, SWK3 cells showed increased proliferation in response to S100A9-GST only, suggesting that Smad4 is important in regulating the proliferative response to exogenous S100A8. Transient Smad4 knockdown in SWD20 and Panc-1 was accompanied by a loss of response to S100A8-GST. Thus, although the measured proliferation effects of S100A8/A9 were not as marked as their chemotactic effects, the dependence on Smad4 for S100A8 signaling was a recurrent theme. Our RAGE-blocking experiments provide evidence that S100A8 and S100A9 activation of the Smad4 pathway occurs at least in part through RAGE. The ability of Advanced Glycation Endproducts to activate transforming growth factor-β signaling via RAGE and mitogen-activated protein kinases has been established by Li *et al.* (29).

Monocytes, once recruited from blood initially express both S100A8 and S100A9 and as they mature, they lose S100A8 expression, leaving only S100A9, which is also subsequently lost as the cell matures further (30). We postulate that myeloid cells (such as monocytes) expressing both S100A8 and S100A9 are recruited to the tumor microenvironment of Smad4-positive tumors, where they secrete S100A8/A9 that may promote further recruitment of inflammatory cells as well as cancer cell growth and invasion. In the case of Smad4-negative tumors, our data suggest that the myeloid cells recruited express S100A9, but exhibit lower S100A8 expression. Alternatively, the myeloid cells recruited are similar in the case of Smad4-positive and Smad4-negative tumors, however, the rate of differentiation, once recruited to the environment of Smad4-negative tumors is different, such that S100A8 expression becomes very transient, leaving relatively greater numbers of cells expressing only S100A9. Either way, our experiments indicate that the cross talk between Smad4-negative cancer cells and myeloid cells occurs in an environment that involves S100A9 to a greater extent than S100A8 and where the cancer cells respond better to S100A9 than S100A8. Further research into S100A8/A9 signaling in the tumor microenvironment will shed light on how these proteins may influence the processes of tumor development/spread and will provide opportunities for targeted intervention.

Supplementary material

Supplementary Figures 1–6 and Table 1 can be found at http://carcin.oxfordjournals.org/

Funding

National Institute for Health Research Liverpool Pancreatic Biomedical Research Unit; Liverpool Experimental Cancer Medicine Centre; Royal College of Surgeons of England, UK; Pancreatic Cancer Research Fund, UK; European Framework 6 Integrated project grant (LSHB-CT-2006-018771).

Acknowledgements

Conflict of Interest Statement: None declared.

References

1. Witz,I.P. (2008) Yin-yang activities and vicious cycles in the tumor microenvironment. *Cancer Res.*, **68**, 9–13.
2. Murdoch,C. *et al.* (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer*, **8**, 618–631.
3. Joyce,J.A. *et al.* (2009) Microenvironmental regulation of metastasis. *Nat. Rev. Cancer*, **9**, 239–252.
4. Sheikh,A.A. *et al.* (2007) The expression of S100A8 in pancreatic cancer-associated monocytes is associated with the Smad4 status of pancreatic cancer cells. *Proteomics*, **7**, 1929–1940.
5. Derynck,R. *et al.* (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nat. Genet.*, **29**, 117–129.
6. Odink,K. *et al.* (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature*, **330**, 80–82.
7. Roth,J. *et al.* (1993) Expression of calcium-binding proteins MRP8 and MRP14 is associated with distinct monocytic differentiation pathways in HL-60 cells. *Biochem. Biophys. Res. Commun.*, **191**, 565–570.
8. Foell,D. *et al.* (2007) S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J. Leukoc. Biol.*, **81**, 28–37.
9. Nacken,W. *et al.* (2003) S100A9/S100A8: myeloid representatives of the S100 protein family as prominent players in innate immunity. *Microsc. Res. Tech.*, **60**, 569–580.
10. Striz,I. *et al.* (2004) Calprotectin—a pleiotropic molecule in acute and chronic inflammation. *Physiol. Res.*, **53**, 245–253.
11. Ehrchen,J.M. *et al.* (2009) The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J. Leukoc. Biol.*, **86**, 557–566.
12. Turovskaya,O. *et al.* (2008) RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. *Carcinogenesis*, **29**, 2035–2043.
13. Hiratsuka,S. *et al.* (2006) Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat. Cell Biol.*, **8**, 1369–1375.
14. Hiratsuka,S. *et al.* (2008) The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat. Cell Biol.*, **10**, 1349–1355.
15. Shekouh,A.R. *et al.* (2003) Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma. *Proteomics*, **3**, 1988–2001.
16. Thompson,C.C. *et al.* (2007) Pancreatic cancer cells overexpress gelsolin family-capping proteins, which contribute to their cell motility. *Gut*, **56**, 95–106.
17. Schwarte-Waldhoff,I. *et al.* (1999) DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression. *Oncogene*, **18**, 3152–3158.
18. Muller,N. *et al.* (2002) Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells. *Oncogene*, **21**, 6049–6058.
19. Jazag,A. *et al.* (2005) Smad4 silencing in pancreatic cancer cell lines using stable RNA interference and gene expression profiles induced by transforming growth factor-beta. *Oncogene*, **24**, 662–671.
20. Gebhardt,C. *et al.* (2008) RAGE signaling sustains inflammation and promotes tumor development. *J. Exp. Med.*, **205**, 275–285.
21. Ghavami,S. *et al.* (2008) S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. *J. Leukoc. Biol.*, **83**, 1484–1492.
22. Stulik,J. *et al.* (1999) The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: the S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis*, **20**, 1047–1054.
23. Roth,J. *et al.* (1993) MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. *Blood*, **82**, 1875–1883.

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24. Funada,Y. *et al.* (2003) Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncol. Rep.*, **10**, 309–313.
25. Khorana,A.A. *et al.* (2003) Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with Stage II and Stage III colon carcinoma: a role for the host response in prognosis. *Cancer*, **97**, 960–968.
26. Forssell,J. *et al.* (2007) High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin. Cancer Res.*, **13**, 1472–1479.
27. Miyaki,M. *et al.* (2003) Role of Smad4 (DPC4) inactivation in human cancer. *Biochem. Biophys. Res. Commun.*, **306**, 799–804.
28. Kitamura,T. *et al.* (2007) SMAD4-deficient intestinal tumors recruit CCR1+ myeloid cells that promote invasion. *Nat. Genet.*, **39**, 467–475.
29. Li,J.H. *et al.* (2004) Advanced glycation end products activate Smad signaling via TGF-beta-dependent and independent mechanisms: implications for diabetic renal and vascular disease. *FASEB J.*, **18**, 176–178.
30. Zwadlo,G. *et al.* (1988) Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin. Exp. Immun.*, **72**, 510–515.

Received February 6, 2010; revised May 29, 2010; accepted June 29, 2010

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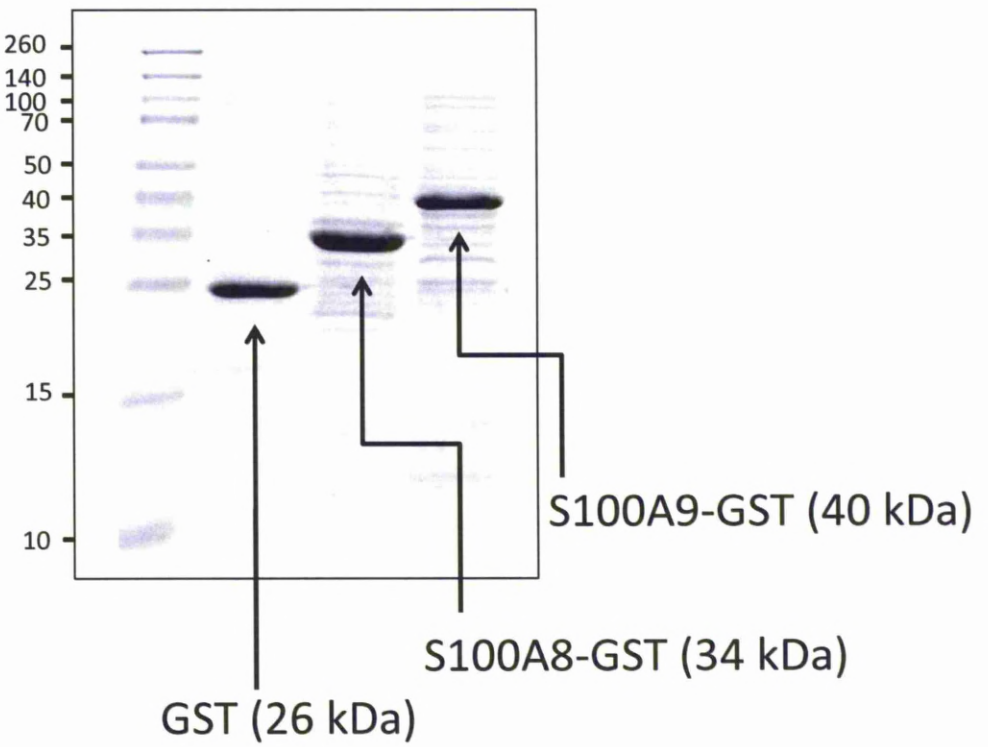

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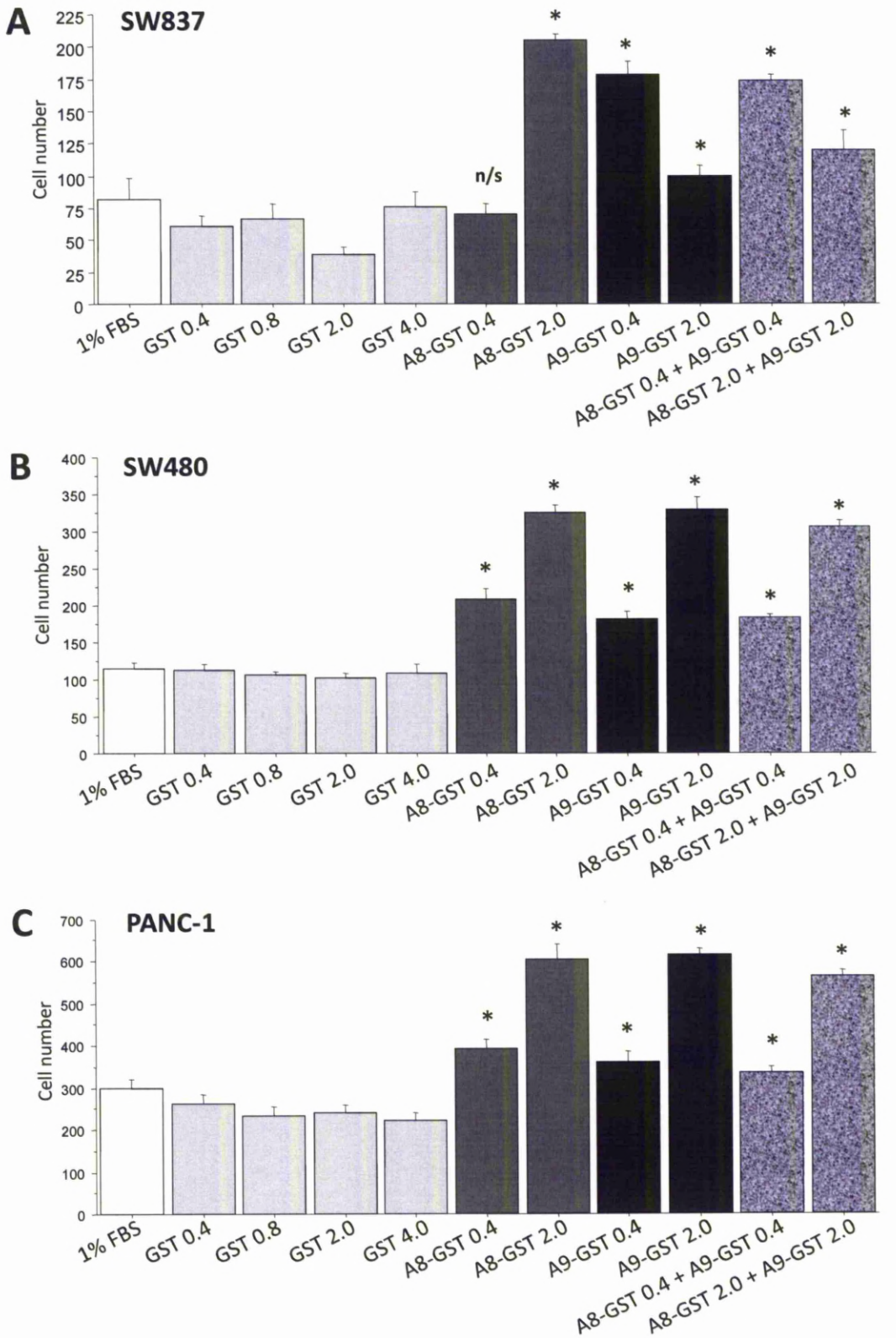

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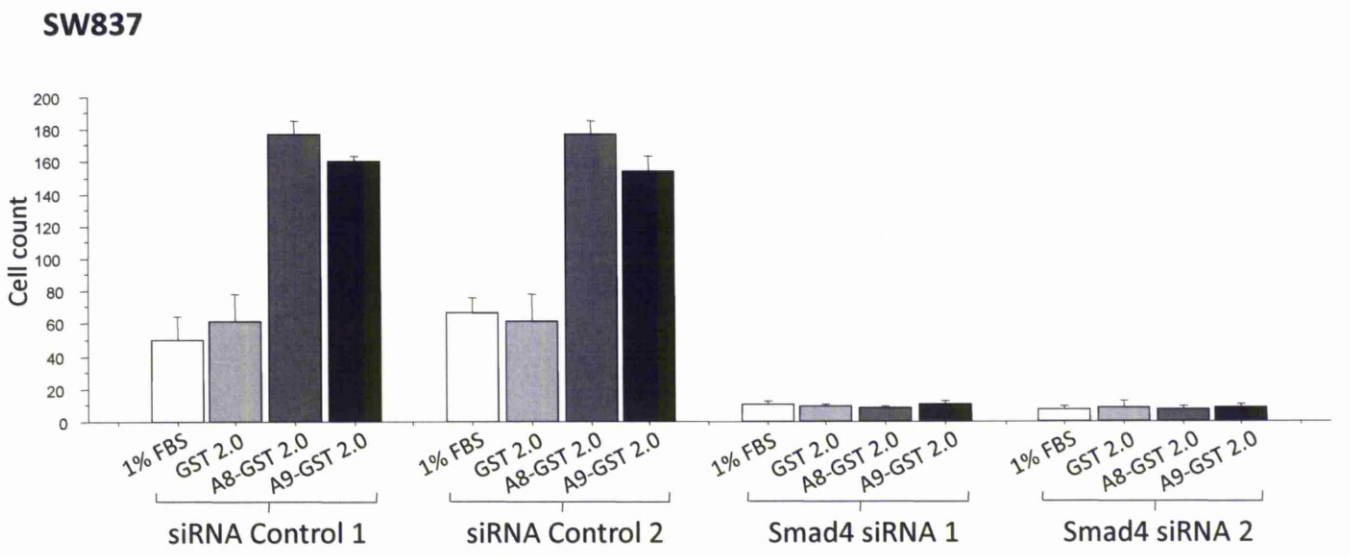

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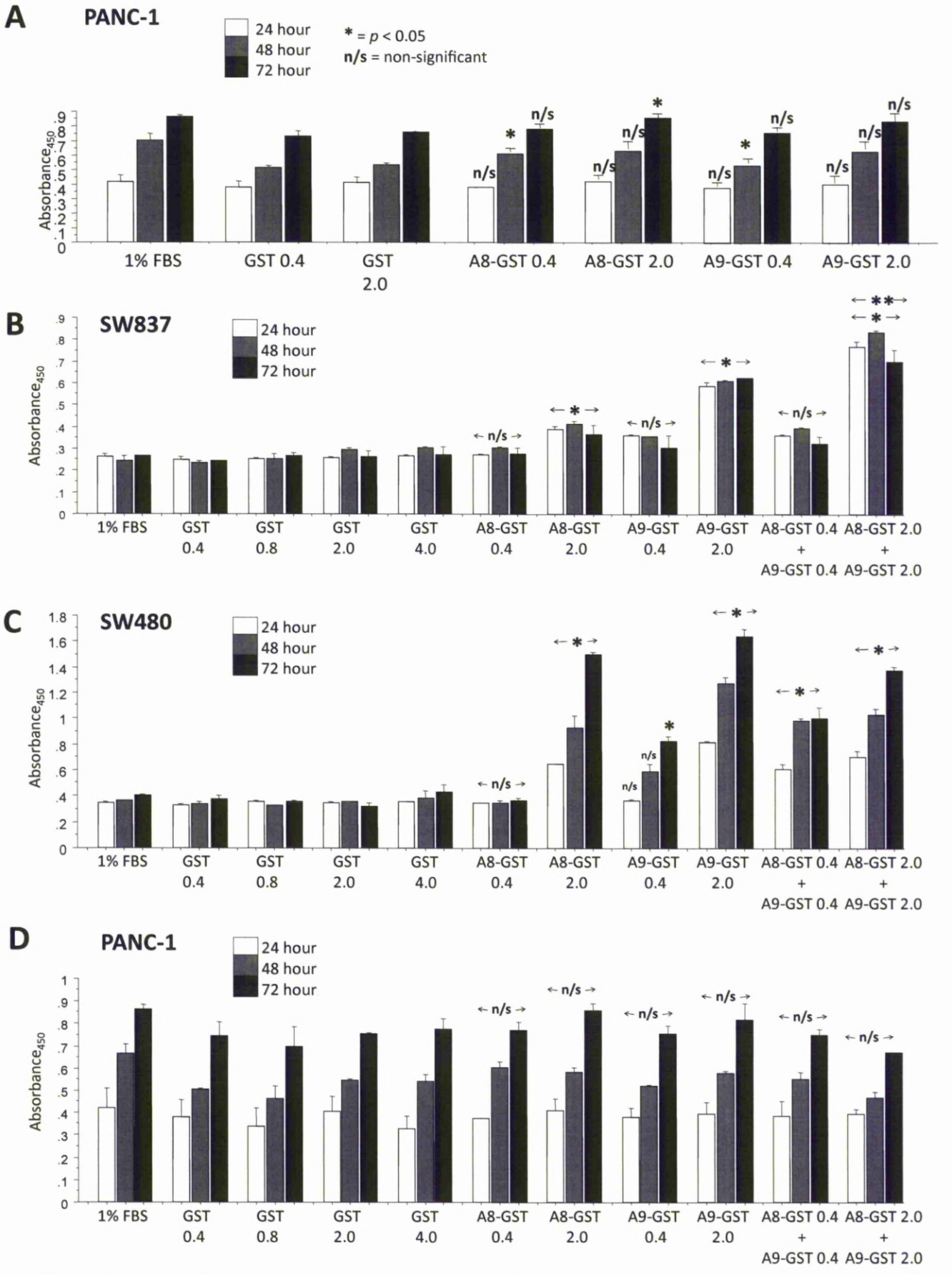

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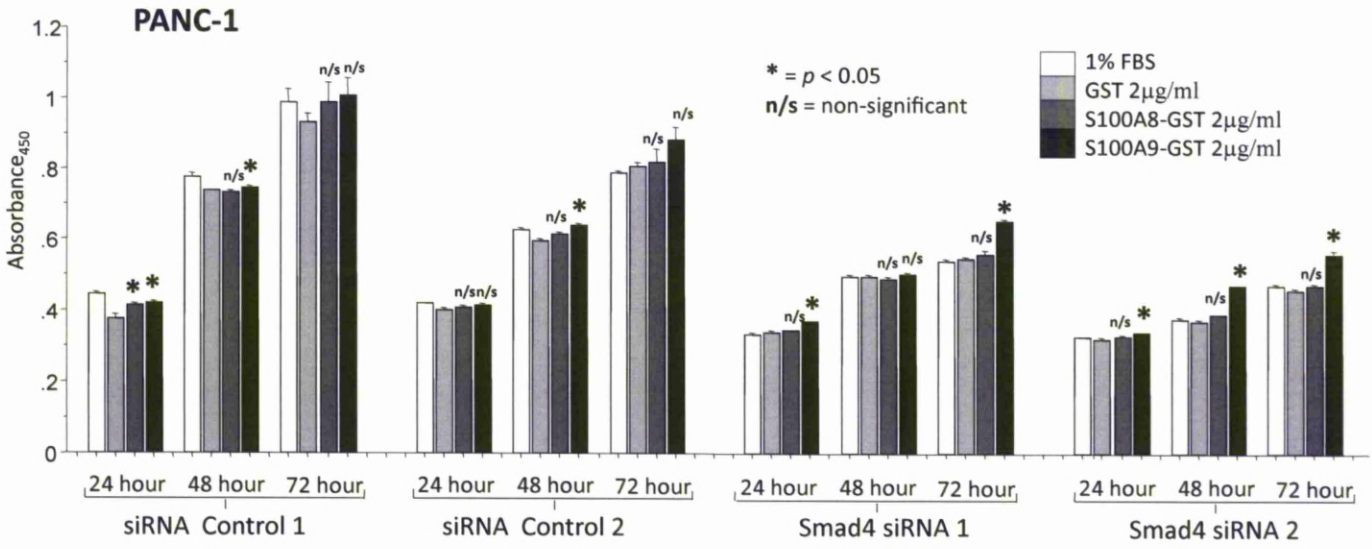

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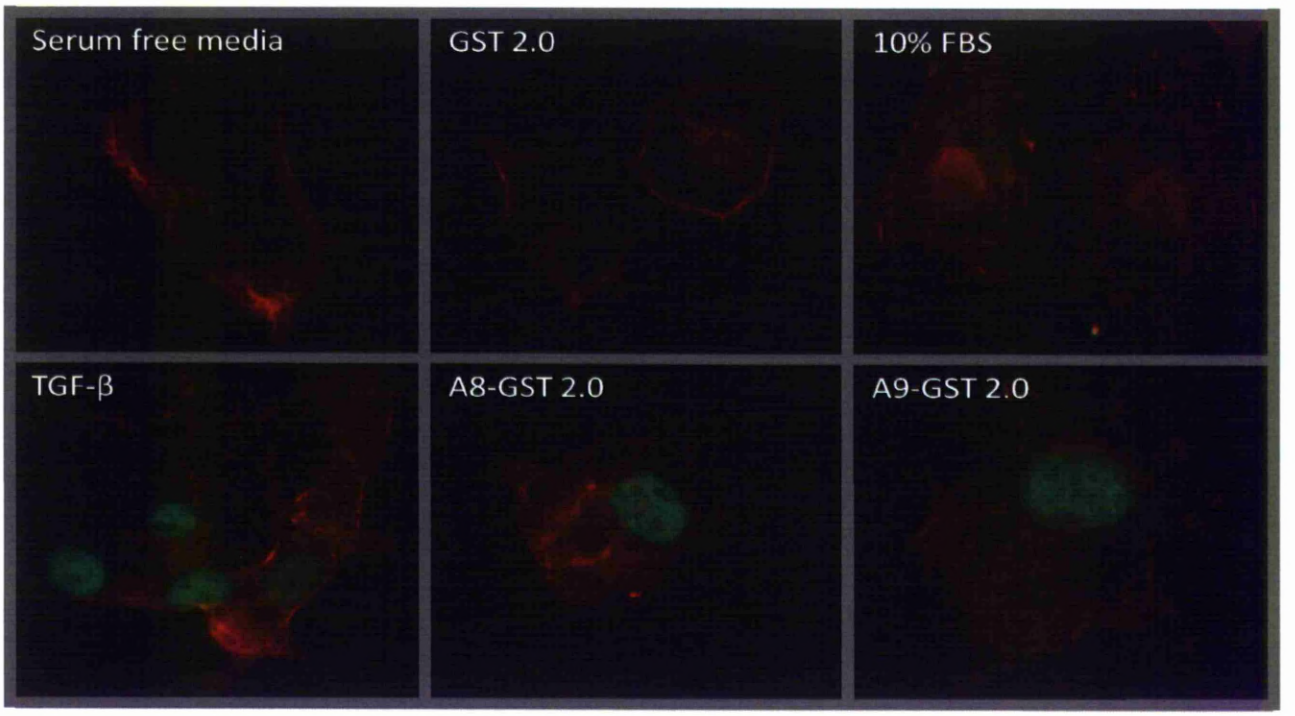

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